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PRINCIPAL INVESTIGATOR: Shiva Shahrara, Ph.D.

CONTRACTING ORGANIZATION: The University of Illinois  
CHICAGO, IL 60612

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| 14. ABSTRACT<br><br>In this proposal we show that ligation of IL-17 to IL-17R plays a critical role in rheumatoid arthritis (RA) joint vascularization. However, less is known about the pro-angiogenic factors downstream of IL-17 cascade that could indirectly contribute to RA angiogenesis. In this progress report, we discovered a novel chemokine (CCL28) and its corresponding receptor (CCR10) that is modulated by IL-17 in RA myeloid cells. We uncover for the first time the expression pattern, regulation and function of this IL-17 downstream pathway. We further provide strong evidence that supports utilizing the CCL28/CCR10 cascade as a potential therapeutic target in RA. These findings were recently published in the highest ranked rheumatology journal (Annals of Rheumatic Disease) and the discoveries were highlighted in 11 other scientific journals.  |             |                         |                            |  |   |
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**The specific aims of this proposal will remain unchanged and will be performed as initially proposed.**

## **INTRODUCTION**

TH-17 cells are a newly discovered CD4 helper T-cells that produce interleukin-17A (also known as IL-17). IL-17 is found in Rheumatoid Arthritis (RA) synovial tissue and fluid, and the percentage of TH-17 cells is significantly higher in RA synovial fluid compared to RA or normal peripheral blood (1, 2). IL-17 has been shown to have a profound effect in experimental models of arthritis however its role in Rheumatoid Arthritis is undefined. Angiogenesis is an early and a critical event in the pathogenesis of RA. Since our preliminary data suggests that IL-17 plays an important role in RA angiogenesis, this grant was proposed to determine the mechanism by which IL-17 induces neovascularization.

Our overriding hypothesis is that IL-17 mediates angiogenesis in RA through activation of the PI3K pathway, and this effect may be dependent or independent of other proangiogenic factors. To test this hypothesis we will investigate the contribution of IL-17 and its receptors to RA synovial fluid-mediated endothelial migration and blood vessel growth. We will also identify signaling pathways that are involved in IL-17-mediated neovascularization and examine whether mice deficient in a particular signaling pathway are unable to mediate neovascularization through IL-17. To further examine the role of IL-17 and its synergistic effect with other factors in inducing HMVEC migration *in vivo*. Additionally, the indirect role of IL-17 in induction of angiogenesis and arthritis will be examined by blocking the effect of relevant IL-17 associated pro-angiogenic factors in *in vitro* and *in vivo* models of angiogenesis and experimental arthritis. The unmet need is to elucidate the mechanisms by which IL-17 mediates angiogenesis in RA and to determine whether targeting IL-17 and/or its intermediary molecules will provide a therapeutic intervention for RA patients.

## **BODY**

In **task 1**, we proposed to examine the mechanism by which IL-17 induces angiogenesis, (time frame, months 1-13).

**A summary of the 2011 progress report:** In our progress report submitted in July of 2011 we addressed the specific aims proposed in Task 1 by demonstrating that ligation of IL-17 to IL-17RC but not IL-17 RA is responsible for the direct effect of IL-17 on angiogenesis. We further documented that ligation of IL-17 to IL-17RC mediates endothelial migration and tube formation via activation of AKT1/PI3K pathway. IL-17 expressed in the joint synovial fluid plays a crucial role in RA pathogenesis since neutralization of IL-17 in the fluid or IL-17RC on endothelial cells markedly reduces endothelial homing and vessel formation. *In vivo* we demonstrate for the first time that local expression of IL-17 induces joint inflammation that is associated with increased vascularization. Confirming these observations we also show that hemoglobin concentration is 10 fold higher in IL-17 containing Matigel plugs compared to PBS controls. These results validate that IL-17 promote RA angiogenesis directly through IL-17RC ligation and activation of AKT1 pathway. These results are published in J Immunol 2010, 184:3233-3241. Most recently others have shown that IL-17 is capable of inducing endothelial cell invasion by activating production of CXCL1/GRO $\alpha$  and MCP-1 from RA synovial tissue fibroblasts suggesting that there may be other mechanisms involved in IL-17 mediated vascularization. Therefore we next studied the indirect role of IL-17 in RA pathology and angiogenesis proposed in aim 3.

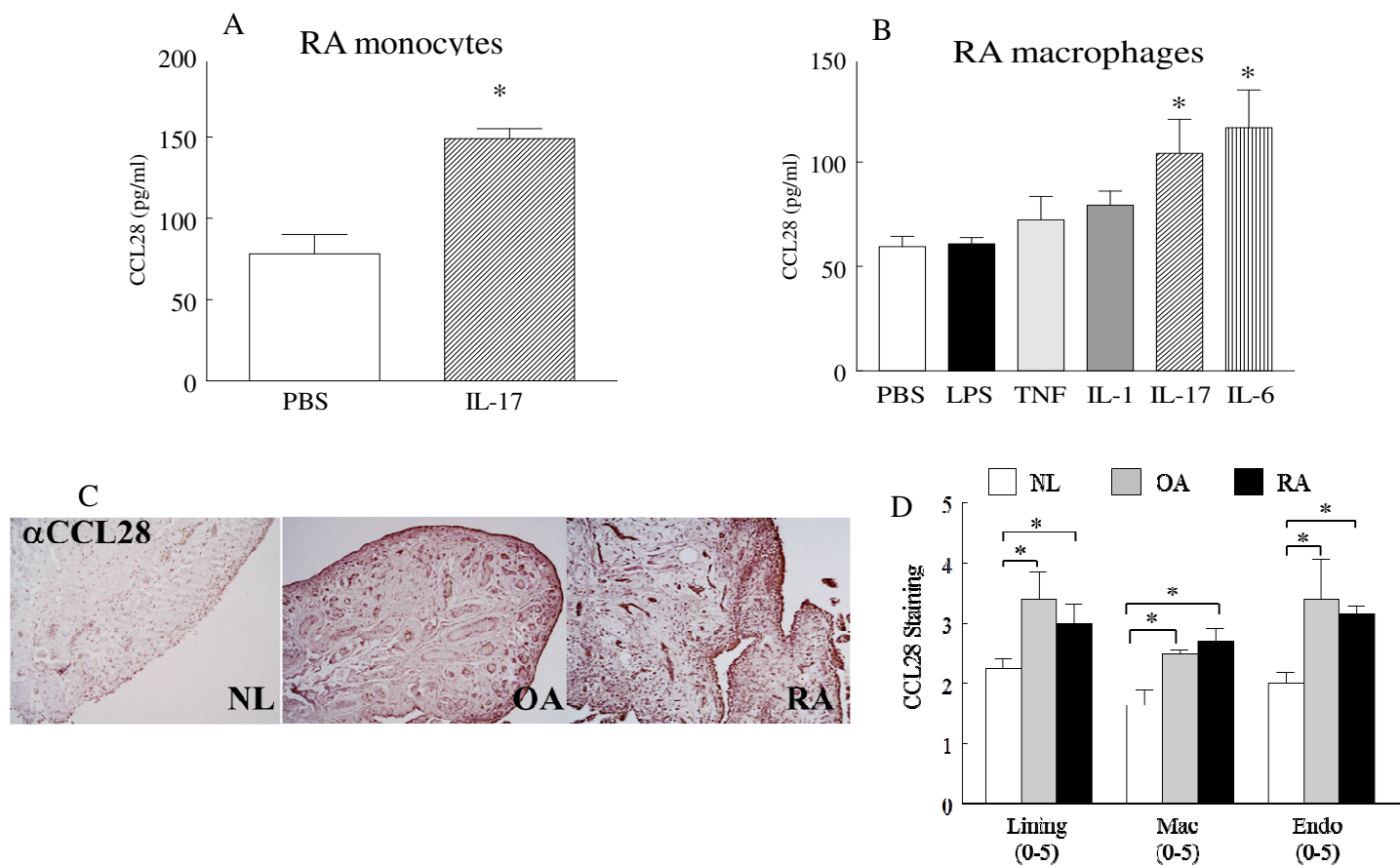


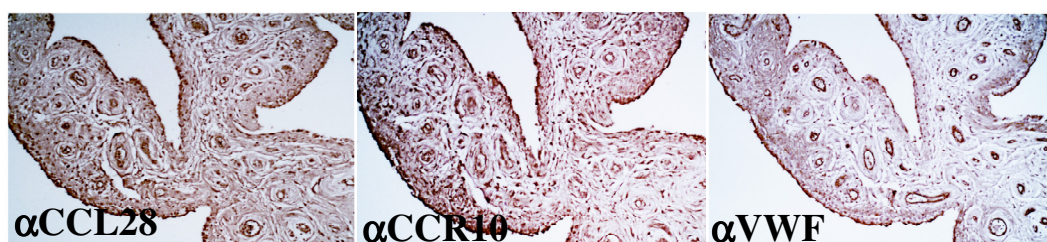
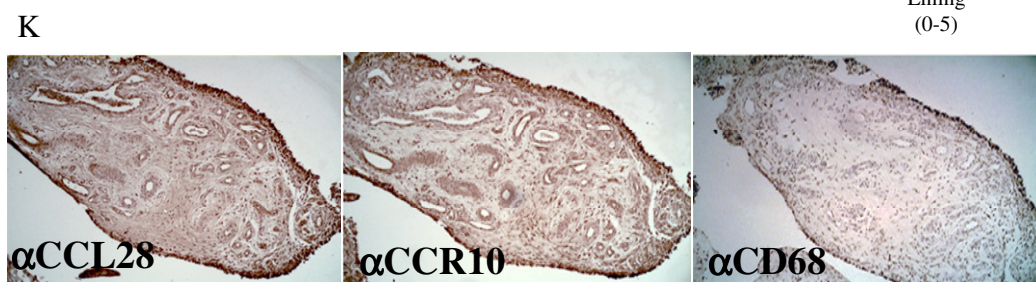
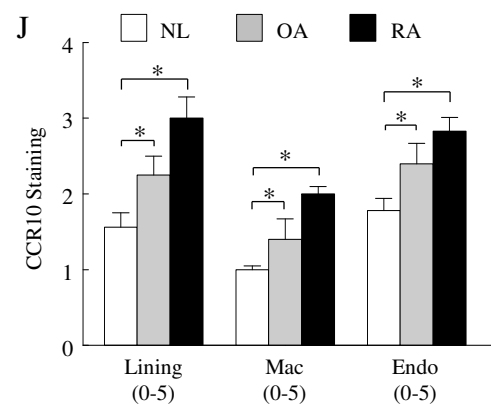
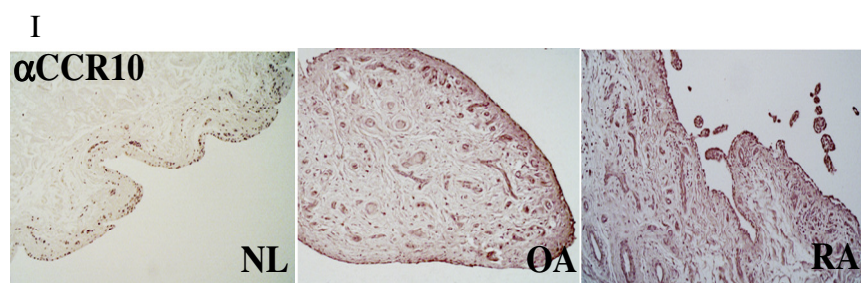
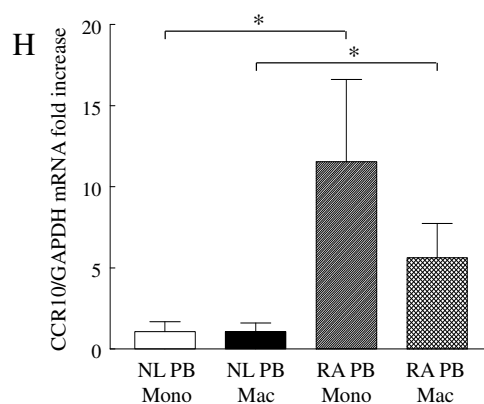
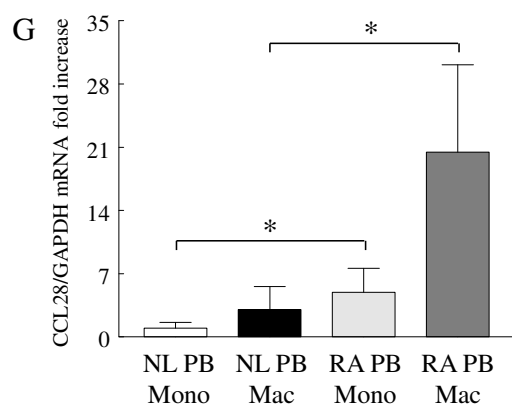
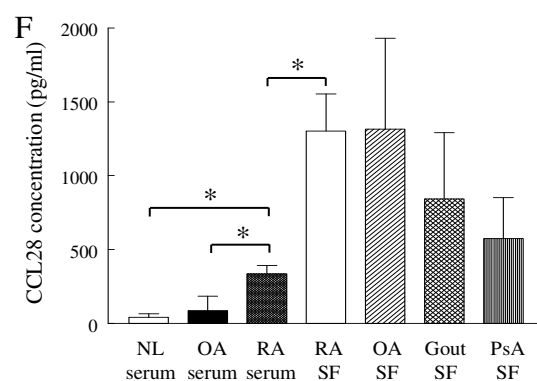
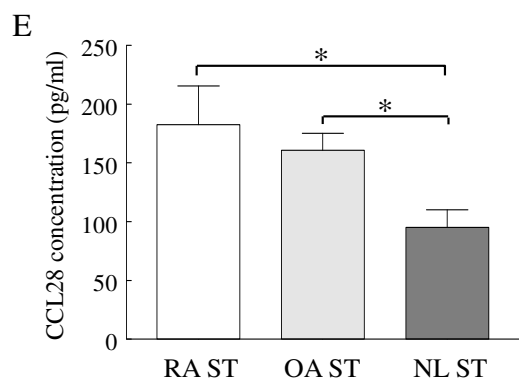
**TASK 2.** To determine whether IL-17 plays a role in endothelial cell homing to RA ST. We will: (time frame, months 14-21).

**2A and 2B. Examine whether IL-17 plays a role in RA ST mediated endothelial cell migration, (time frame, months 14-18).**

We found that myeloid cells in RA synovial tissue and RA blood activated by IL-17 produce a novel and unidentified factor called Mucosa associated Epithelial Chemokine (MEC) or CCL28 (Figs. 1A-B). Next expression pattern and mechanism of function of CCL28 and its receptor CCR10 was investigated in RA.

When expression of CCL28 and CCR10 was examined in RA compared to OA and NL ST, we found that individuals with RA and OA have markedly higher CCL28 and CCR10 expression levels on ST macrophages and endothelial cells (Figs. 1C-D, 1I-J). Next, colocalization of CCL28 and CCR10 on RA ST myeloid cells and blood vessels was validated by serial section staining of CCL28 and CCR10 to CD68 and VWF positive cells (Figs. 1K). Consistent with our histological studies, we document that patients with arthropathies express elevated levels of CCL28 in their ST (RA and OA) and SF [RA, OA, gout, psoriatic arthritis (PsA)] compared to NL individuals (assessed using ST or sera) (Figs. 1E-F). These results suggest that in patients with joint related disease, the inflammatory response may contribute to production of CCL28 from myeloid and endothelial cells relative to normal controls. Interestingly we found that both CCL28 and CCR10 transcription levels are increased in RA compared to NL myeloid cells. While RA macrophages differentiated from monocytes exhibit a greater trend for CCL28 expression, CCR10 expression trend is higher in RA monocytes compared to differentiated macrophages (Figs. 1G-H).



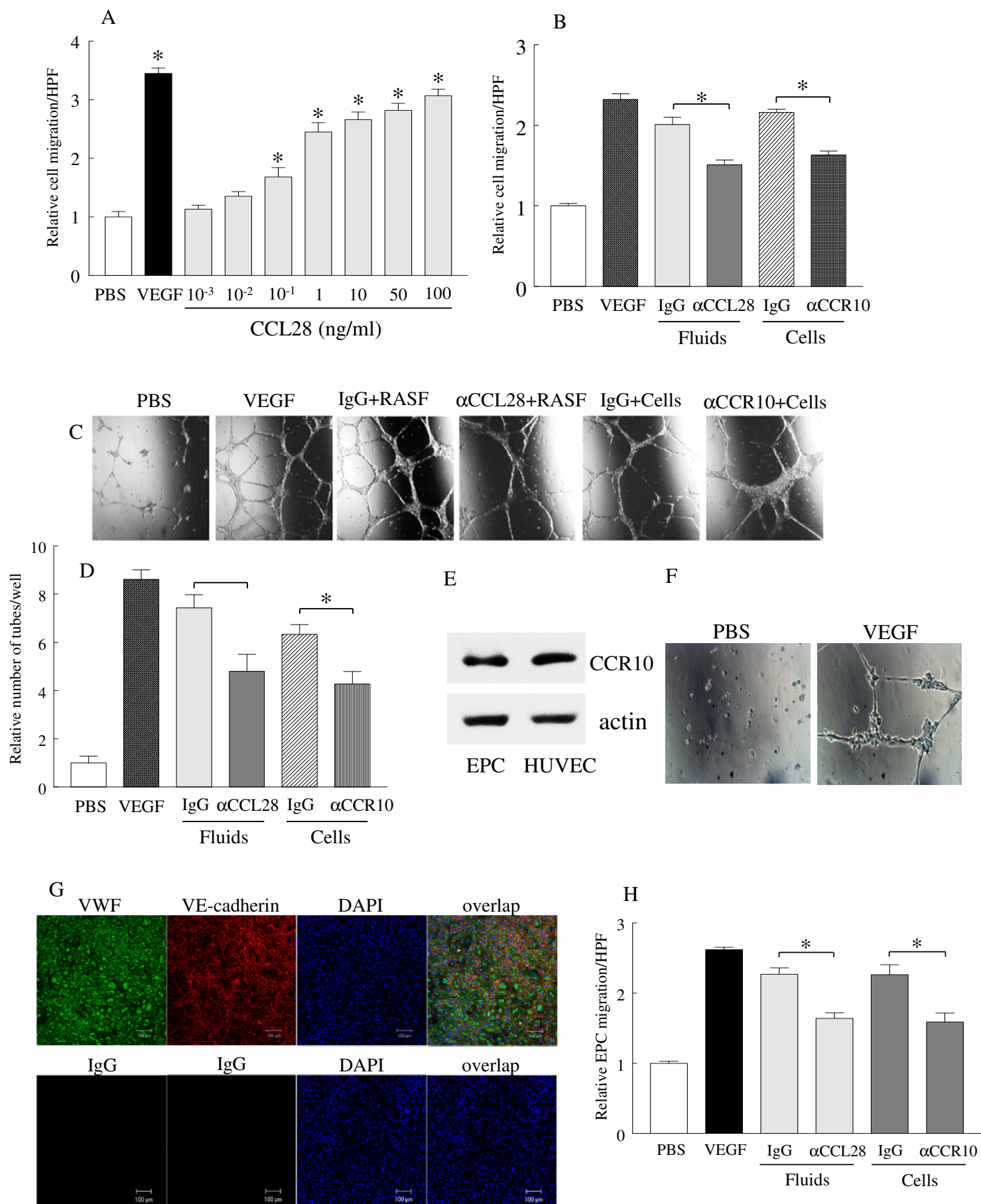


**Figure 1. CCL28 and CCR10 are elevated on RA ST myeloid and endothelial cells and myeloid CCL28 expression is modulated by IL-17 stimulation.** RA monocytes (A) and RA PB in vitro differentiated macrophages (B) were either untreated or stimulated by 10 ng/ml LPS, TNF- $\alpha$ , IL-1 $\beta$ , IL-6 or 50ng/ml IL-17 for 24-72h prior to determining protein levels of CCL28 by ELISA. NL, OA and RA ST were stained with anti-human CCL28 (C) or CCR10 (I) (original magnification  $\times$  200). Positive immunostaining of CCL28 (D) and CCR10 (J) were scored on a 0-5 scale in ST lining, sublining macrophages (Mac) and endothelial cells (Endo) and staining is shown as mean  $\pm$  SEM, n=10. E. CCL28 protein levels were quantified in NL, OA and RA STs by ELISA, n=10. F. Protein concentration of CCL28 was assessed in sera obtained from RA (n=22), OA (n=7) and NL (n=19) and SF procured from RA (n=22), OA (n=10), gout (n=10) and PsA (n=10) patients. Expression levels of CCL28 (G) or CCR10 (H) were quantified in NL and RA monocytes (Mono) and PB differentiated macrophages (Mac) by real time RT-PCR which were normalized to GAPDH and the values are shown as fold increase above NL PB monocytes, n=12-15. K. Colocalization of CCL28 and CCR10 on macrophages (CD68+) and endothelial cells (VWF+) was examined when RA serial sections were stained with anti-CCL28, anti-CCR10, anti-CD68 and anti-VWF antibodies (original magnification  $\times$  200). Values are the mean  $\pm$  SE. \* represents p <0.05.

To our knowledge, we show for the first time that CCL28 and CCR10 expression is elevated in RA and OA ST compared to NL ST myeloid and endothelial cells. Yet, upregulation of CCL28 has been reported in the epithelial cells of inflamed mucosal tissue detected in colons, duodenal mucosa, ileum of patients with Cohn's disease, lungs and liver bile ducts as well as in the skin epithelial cells of patients with psoriasis and atopic dermatitis (3-7). In normal physiological conditions, CCL28 is predominately produced from epithelial cells present in small and large intestines, reproductive tracts, lungs, lactating mammary glands and salivary glands as well as from pulmonary dendritic cells (7-9). Moreover the CCL28 corresponding receptor CCR10 is expressed in T cells, IgA antibody secreting plasma cells, Langerhans cells and melanocytes (10, 11). Accentuated levels of CCL28 were detected in ST or SF of arthritis patients (RA, OA, gout and PsA) compared to normal individuals, indicating the importance of CCL28/CCR10 cascade in inflammation. Although comparable levels of CCL28 were detected in RA and OA ST and SF, CCL28 concentrations were markedly lower in OA compared to RA sera. Therefore studies were conducted to examine the mechanism by which CCL28 and CCR10 promote pathogenesis in RA myeloid and endothelial cells. Interestingly we show that differentiated macrophages demonstrate a greater trend of CCL28 expression compared to monocytes, highlighting the significance of CCL28 function in the RA joints rather than in the circulating blood cells. On the contrary, the differentiation process reduces CCR10 expression trend in macrophages. These findings suggest that the circulating monocytes may require higher levels of CCR10 to respond to the CCL28 produced by joint cells, however once these CCR10+ monocytes reach their destination the differentiation process results in a marginal CCR10 downregulation.

#### ***Ligation of joint CCL28 to endothelial CCR10 facilitates RA SF mediated angiogenesis***

Since similar to RA, CCR10 was highly expressed in endothelial progenitor cells (EPC)s and HUVECs (Fig. 2E), these cells were used as substitutes for RA endothelial cells and their role was investigated in CCL28 mediated RA angiogenesis. We uncovered that CCL28 can strongly attract endothelial cells starting at 0.1 ng/ml, indicating that CCL28 (up to 3300 pg/ml expressed in RA SF) can contribute to endothelial cell migration at a physiologically relevant concentration (Fig. 2A). We further document that ligation of SF CCL28 to endothelial CCR10 is involved in RA angiogenesis, as neutralization of CCL28 in RA SF or blockade of CCR10 on HUVECs or EPCs can significantly reduce RA SF driven endothelial chemotaxis or tube formation by 30-40% (Figs. 2B-D, 2H). These results suggest that CCL28 at concentrations present in the RA joint can foster endothelial infiltration and capillary tube formation through CCR10 ligation; hence supporting the importance of CCL28/CCR10 cascade in RA angiogenesis.



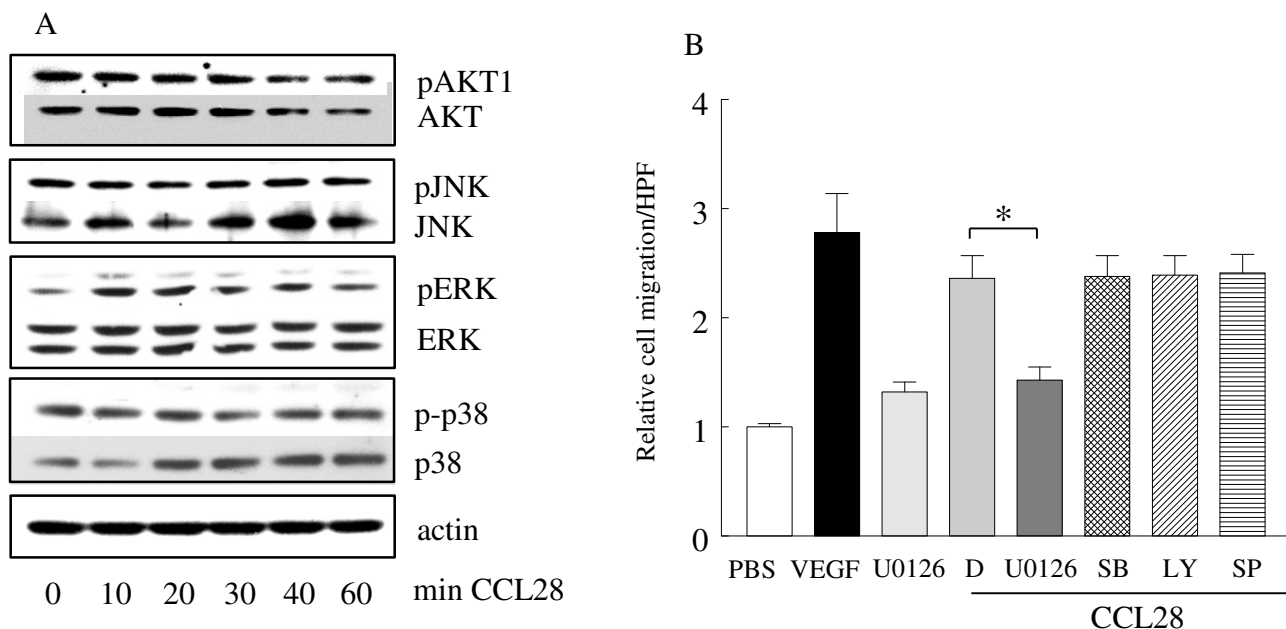


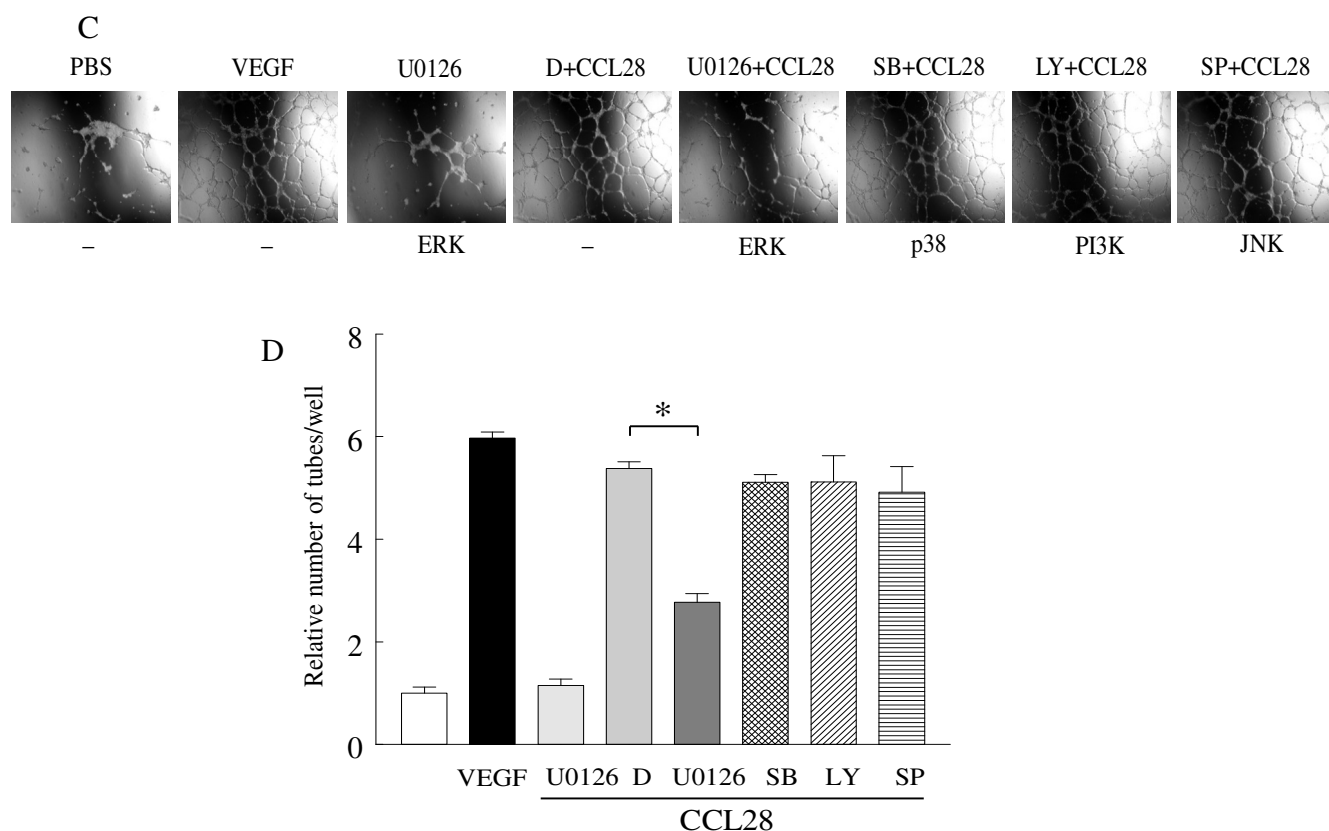
**Figure 2. CCL28 strongly promotes EPC migration and capillary tube formation through CCR10 binding.**

**A.** Dose-response curve of CCL28-induced HUVEC chemotaxis was performed in a Boyden chemotaxis chamber with varying concentrations (0.001-100 ng/ml),  $n=3$ . Anti-CCL28 (10  $\mu\text{g/ml}$ ) or control IgG was added to RA SFs (1:20) or HUVECs were incubated with antibodies to CCR10 (10  $\mu\text{g/ml}$ ) or isotype control for 1h prior to performing chemotaxis (**B**) or tube formation (**D**) in response to RA SFs,  $n=8$ . **C.** Photomicrographs were taken of representative wells treated with PBS, VEGF (10 ng/ml), IgG or anti-CCL28 (10 $\mu\text{g/ml}$ ) plus RA SFs (1:20) and IgG or anti-CCR10 (10 $\mu\text{g/ml}$ ) plus HUVECs. **E.** Expression levels of CCR10 and equal actin loading was assessed in EPCs and HUVECs by Western blot analysis,  $n=3$ . **F.** The ability of EPCs to form capillary tubes was tested in response to PBS and VEGF (10 ng/ml),  $n=3$ . **G.** To validate EPC purity, cells were stained with endothelial markers VWF (1:1000), VE-cadherin (1:200) or IgG control and positive staining was overlapped on DAPI+ cells (1:1000) (original magnification  $\times 400$ ),  $n=3$ . **H.** Anti-CCL28 (10  $\mu\text{g/ml}$ ) or control IgG was added to RA SFs (1:20) additionally EPCs were incubated with antibodies to CCR10 (10  $\mu\text{g/ml}$ ) or isotype control for 1h prior to performing chemotaxis,  $n=3$ . Endothelial cell chemotaxis or tube formation is shown as a relative value and is normalized to PBS values. Values are the mean  $\pm$  SE. \* represents  $p < 0.05$ .

***CCL28 driven angiogenesis is interconnected to ERK signaling***

To determine which endothelial signaling pathways are activated by CCL28, phosphorylation of the MAPK and AKT pathways was determined by immunoblot analysis. Our data demonstrate that CCL28 strongly phosphorylates ERK (at 10 and 100 ng/ml) however AKT1, p38 and JNK pathways were not affected by this process (Figs. 3A). To determine which signaling pathway contributes to CCL28 induced endothelial migration and capillary formation, chemical inhibitors at concentrations of 5  $\mu\text{M}$  were utilized, as 10  $\mu\text{M}$  was toxic and resulted in cell death. As expected, inhibition of PI3K/AKT, JNK and p38 was ineffective in suppressing CCL28 induced endothelial chemotaxis and tube formation, while inhibition of ERK cascade markedly reduced both functions by 30-40% (Fig. 3B-D). These results suggest that joint CCL28 promotes RA angiogenesis through activation of ERK pathway.

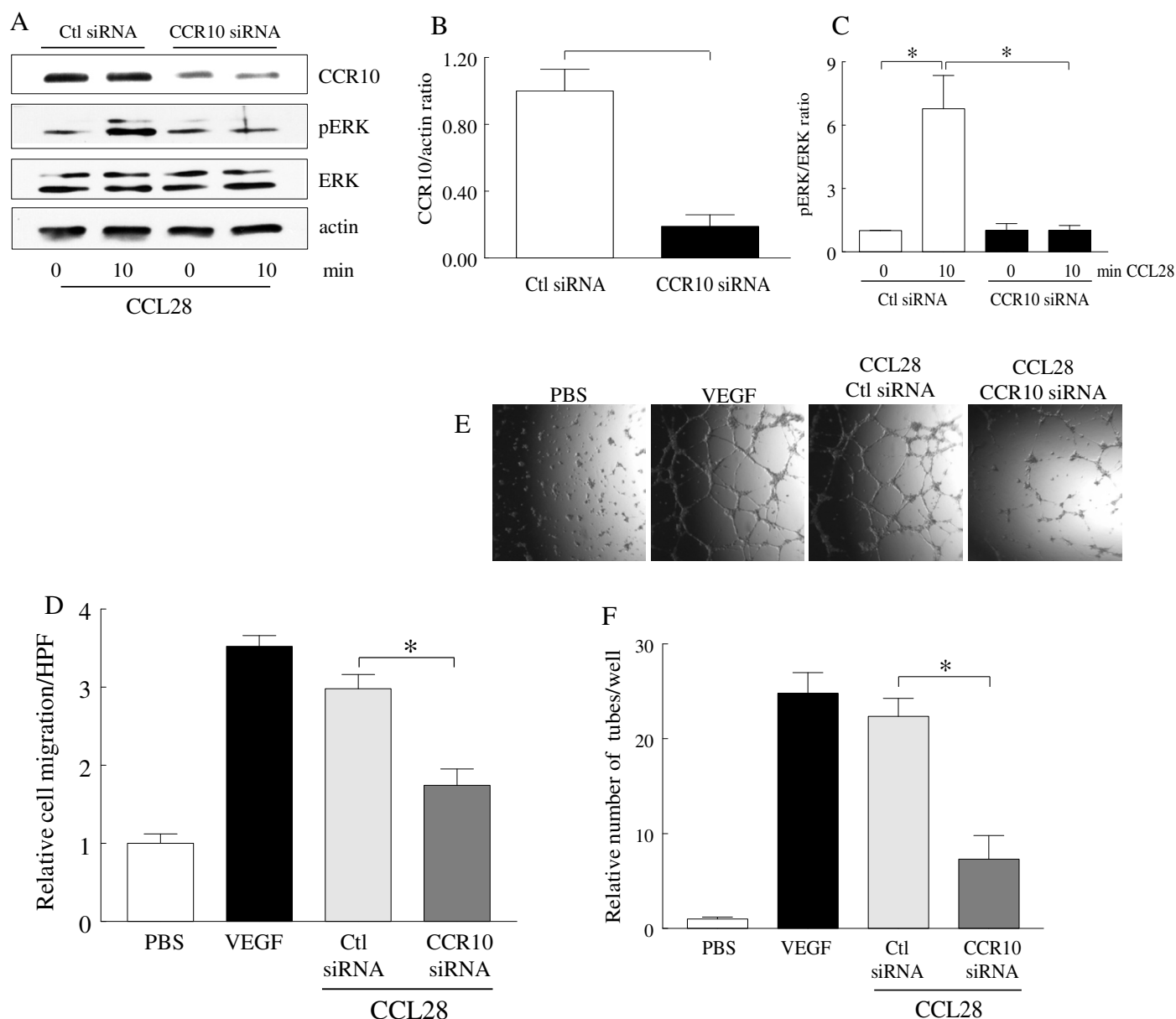




**Figure 3. CCL28 driven endothelial migration and tube formation is facilitated by ERK activation.** **A.** Cells were stimulated with CCL28 (100 ng/ml) for 0-60 minutes and thereafter cell lysates were probed for pAKT1, pJNK, pERK, p-p38 or equal loading controls, n=4-6. HUVECs were incubated with DMSO or inhibitors (5  $\mu$ M) to ERK (U0126; with or without CCL28 addition), p38 (SB203580), PI3K (LY294002), JNK (SP600125) for 45 min prior to performing endothelial chemotaxis (**B**) or tube formation (**D**) in response to CCL28 (50 ng/ml), n=3. **C.** Photomicrographs were taken of representative wells treated with PBS, VEGF (10 ng/ml), U0126, DMSO plus CCL28 (100 ng/ml), U0126 plus CCL28, SB203580 (SB) plus CCL28, LY294002 (LY) plus CCL28, SP600125 (SP) plus CCL28.

### ***Activation of ERK pathway is linked to ligation of CCL28 to endothelial CCR10 and RA angiogenesis***

Since phosphorylation of ERK is pivotal for CCL28 induced chemotaxis and tube formation, we next asked whether ligation of CCR10 is interconnected to CCL28 activation of ERK pathway. Employing CCR10 knockdown cells which had 80% lower CCR10 expression, we found that while CCL28 driven ERK phosphorylation was abolished compared to control cells, total ERK signaling was unaffected in this process (Figs. 4A-C). Consistent with this notion, CCR10 knockdown cells showed significantly reduced chemotaxis (40%) and tube formation (70%) in response to CCL28 compared to the control cells, suggesting that the proangiogenic effect of CCL28 is attributable to CCR10 ligation (Figs. 4D-F). Collectively these results suggest that ERK is indispensable for CCR10 ligation of CCL28 and angiogenesis mediated by this factor.



**Figure 4. Angiogenesis fostered through ligation of CCL28 to CCR10 is interconnected to ERK signaling.**

**A.** HUVECs transfected with CCR10 specific and nonspecific control siRNA (100 nM) were either untreated or treated with CCL28 (100 ng/ml) for 10 min. Thereafter cell lysates were examined for CCR10, pERK, ERK and actin. Densitometric analysis of the CCR10/actin (**B**) and pERK/ERK (**C**) was performed using Image J software, n=3. CCL28 (50ng/ml) mediated HUVEC chemotaxis (**D**) and tube formation (**F**) was assessed in control and CCR10 knockdown cells, n=3. **E.** Photomicrographs were taken of representative wells treated with PBS, VEGF (10 ng/ml), control (Ctl) knockdown cells plus CCL28 (50 ng/ml) and CCR10 knockdown cells plus CCL28 (50 ng/ml).

We uncovered that EPCs similar to RA joint endothelial cells express high levels of CCR10 and can form capillaries in response to proangiogenic factors. Corroborating with these observations, others have shown that EPCs have a greater ability to migrate into RA ST compared to NL ST implants and the number of infiltrated EPCs is markedly elevated in collagen antibody induced arthritis (CAIA) synovium (12); therefore EPCs were used as surrogates for RA endothelial cells. Our research team has pioneered the field by demonstrating that the production of CCL28 from joint myeloid and endothelial cells is a strong promoter of angiogenesis in EPCs at a physiologically relevant concentration. We further show that both CCL28 and CCR10 are involved in RA SF mediated EPC chemotaxis. Moreover our data document that angiogenesis facilitated through ligation of CCL28 to CCR10 is interconnected to ERK signaling as CCR10 knockdown cells demonstrate dysfunctional CCL28 induced ERK signaling and chemotaxis. To our knowledge this is the first evidence that shows a direct role for CCL28 and CCR10 in angiogenesis. A recent study found that in ovarian tumor cells, hypoxia triggered CCL28 production, promotes homing of CCR10+ Tregs into the ovarian tumor (13). Interestingly the authors further report that secretion of VEGF from CCR10+ Tregs contributes to ovarian tumor angiogenesis. In this model, Tregs are integral for VEGF production as anti-CCR10 or anti-CD25 (markers for Tregs) treatment greatly suppresses tumor vascularity and VEGF levels (13). Although both we and others demonstrate that angiogenesis can be fostered by ignition of CCL28/CCR10 cascade, there are a number of dissimilarities between these two studies. While we document that CCL28 can directly mediate neovascularization by attracting CCR10+ endothelial cells, the study by Facciabene and colleagues show that CCL28 ligation to Tregs is indirectly responsible for tumor angiogenesis.

We uncovered a novel ligand and receptor pair whose expression was neither described in the cell types identified in this study nor in RA. The current findings unravels that in **RA myeloid cells IL-17 can regulate expression** of the novel CCL28/CCR10 cascade that play an important role in RA vascularization.

**TASK 3 :** To examine the indirect role of IL-17 in mediating angiogenesis and arthritis. We will: (time frame, months 24-36).

**3a. Examine the mechanism by which IL-17-induced angiogenesis is associated with VEGF.** (time frame, months 24-26).

**A summary of the 2013 progress report:** Angiogenesis is an early and a critical event that fosters chronic inflammation and bone erosion in RA by facilitating unbalanced leukocyte migration and pannus formation. Hence inhibition of angiogenesis may lead to identifying novel therapeutic approaches for RA. Macrophages are hypoxia sensors that initiate and maintain angiogenesis in RA synovium. We found that CCR7 was the most highly upregulated gene in macrophages obtained from RA synovial fluid compared to the normal myeloid cells by microarray analysis. In validation of the microarray data, macrophages in the RA synovial tissue lining and endothelial cells in the sublining express elevated levels of CCR7 and its ligand CCL21. We uncovered that synovial CCL21 but not CCL19 is a novel and potent chemoattractant for CCR7+ endothelial cells, which plays a pivotal role in RA tube and blood vessel formation. **In RA synovial tissue explants, CCL21 driven angiogenesis can be also induced indirectly through VEGF production. Interestingly we uncovered that CCL21 is the missing link between IL-17 and VEGF mediated vascularization as blockade of CCL21 function markedly suppresses IL-17 induced VEGF expression in RA ST fibroblasts.**



**3b and 3c. Investigate whether IL-17 angiogenesis *in vivo* is due to downstream proangiogenic factors. (time frame, months 26-36).**

**A summary of the 2012 progress report:** Results from our previous studies demonstrate that IL-17 is a potent proangiogenic factor in RA that can facilitate neovascularization directly through IL-17RC ligation (14). Findings from our (15) and other laboratories indicate that they may be factors downstream IL-17 that also contribute to RA angiogenesis.

To demonstrate whether there are indirect pathways associated with IL-17 induced angiogenesis, we examined potent proangiogenic factors induced by IL-17 in RA synovial tissue as well as in IL-17 experimental arthritis model. We found that expression of CXCL1 and CXCL5 was highly elevated by IL-17 in RA synovial tissue explants and animal models of RA. To demonstrate the pathologic role of CXCL1 and CXCL5 in IL-17 mediated arthritis, neutralizing antibodies to each chemokine were employed. We found that arthritis severity and vascularization were significantly reduced in the anti-CXCL5 treatment group. In contrast, anti-CXCL1 treatment had no effect on IL-17 mediated disease activity or neovascularization, while being capable of inhibiting CXCL1 mediated endothelial chemotaxis *in vitro*. The combination of anti-CXCL1 and anti-CXCL5 was not more effective than anti-CXCL5 treatment alone. We next demonstrated that ligation to CXCR2 facilitates CXCL1 and CXCL5 induced endothelial migration although downstream signaling pathways are differentially regulated by these chemokines. We show that while CXCL1 can induce endothelial migration through activation of PI3K pathway, this process was mediated by CXCL5 through NF- $\kappa$ B signaling. Since both CXCL1 and IL-17 can mediate angiogenesis through the same pathway, blocking of CXCL1 is ineffective in this process whereas suppression of CXCL5 can effectively reduce NF- $\kappa$ B mediated angiogenesis. In conclusion, these observations suggest that IL-17 mediated joint vascularization may be in part due to CXCL5 induction. These findings are now published in *Angiogenesis* 2011; 14: 443-455 (15).

## **KEY RESEARCH ACCOMPLISHMENTS**

Within this short time frame we have shown that:

- RA synovial fluid-induced HMVEC chemotaxis is mediated by IL-17 and IL-17 does not synergize with VEGF in RA synovial fluid induced HMVEC migration.
- Neutralization of IL-17RC but not IL-17RA was involved with suppressing RA synovial fluid-mediated HMVEC migration.
- Activation of PI3K is responsible for IL-17-mediated HMVEC tube formation and migration.
- Expression of CXCL1 and CXCL5 is highly elevated in RA synovial tissues treated with IL-17 and in IL-17 induced arthritis model while VEGF was not markedly increased in any of the mentioned models.
- Inhibition of CXCL5 but not CXCL1 relieves IL-17-induced arthritis.
- Anti-CXCL5 reduces levels of joint TNF- $\alpha$  and vascularization in IL-17-induced arthritis model.

- CXCL5 but not CXCL1 induces endothelial migration and angiogenesis through an IL-17 non overlapping mechanism.
- IL-17 mediated joint vascularization is in part due to CXCL5 induction.
- CCL21 ligation to CCR7 connects IL-17 and VEGF induced neovascularization.
- Disruption of CCL21 binding to CCR7 markedly suppresses IL-17 mediated VEGF transcription.
- IL-17 modulates expression of CCL21 and CCR7 in human endothelial cells.
- CCL21 synergizes with VEGF in promoting endothelial migration.
- Local expression of CCL21 promotes joint inflammation and neovascularization.
- IL-17 regulates expression of CCL28 in RA monocytes and RA peripheral blood in vitro differentiated macrophages
- CCL28 is expressed in in RA synovial fluid and strongly promotes RA angiogenesis through CCR10 ligation and ERK activation.

## REPORTABLE OUTCOMES

### Publications:

1. Pickens SR, Volin MV, Mandelin II AM, Kolls JK, Pope RM, **Shahrara S**. IL-17 Contributes to Angiogenesis in Rheumatoid Arthritis. J. Immunol. 2010, 184; 6 :3233-3241.
2. **Shahrara S**, Pickens SR, Mandelin AM, Huang Q, Karpus W, Kolls JK, Pope RM. IL-17-mediated monocyte migration is partially through CCL2/MCP-1 induction. J. Immunol. 2010, 184; 8:4479-4487.
3. Ruth RH, Park CC, Amin MA, Lesch C, Marotte H, **Shahrara S** and Koch AE. IL-18 as an *in vivo* Mediator of Monocyte Recruitment in Rodent Models of Rheumatoid Arthritis. Arthritis Res. Ther. 2010, 16; 12: R118.
4. Thumbikat P, **Shahrara S**, Sobkoviak R, Done J, Pope RM and Schaeffer AJ. Prostate secretions from men with CP/CPPS inhibits proinflammatory mediators. J. Urol. 2010, 184:1536-42.
5. Pickens SR, Chamberlain ND, Volin MV, Pope RM, Mandelin II AM, **\*Shahrara S**: Characterization of CCL19 and CCL21 in rheumatoid arthritis: Arthritis Rheum 2011, 63: 914–922.
6. Pickens SR, Chamberlain ND, Volin MV, Mandelin AM 2nd, Agrawal H, Matsui M, Yoshimoto T, **\*Shahrara S**. Local expression of IL-27 ameliorate collagen induced arthritis: Arthritis Rheum 2011, 63:2289-98.

**Editorial commented on our paper. Fearon U. “IL-27: a master regulator of inflammation” Arthritis Rheum 2011, 63:2157-2160.**

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#### Findings highlighted in:

1. UIC <http://news.uic.edu/molecules-involved-in-rheumatoid-arthritis-angiogenesis-identified>
2. Arthritis Research UK <http://www.arthritisresearchuk.org/news/general-news/2014/may/new-protein-discovery-sheds-light-on-rheumatoid-arthritis-progression.aspx>
3. Science News Line Medicine <http://www.sciencenewsline.com/articles/2014051615180018.html>
4. National Pain report <http://americannewsreport.com/nationalpainreport/two-proteins-linked-to-rheumatoid-arthritis-8823861.html>

5. Science Daily <http://www.sciencedaily.com/releases/2014/05/140516111003.htm>
6. Arthritis Digest UK <http://arthritisdigest.co.uk/potential-avenue-of-treatment-for-rheumatoid-arthritis-thanks-to-discovery-of-a-new-protein/>
7. US daily news <http://www.dailynews.com/science/molecules-involved-in-rheumatoid>
8. Science Daily Article: <http://www.sciencedaily.com/releases/2014/05/140516111003.htm>
9. Bioscience Technology <http://www.biosciencetechnology.com/news/2014/05/two-proteins-play-key-role-rheumatoid-arthritis>
10. Protein Products online <http://www.ptproductsonline.com/2014/05/protein-molecules-part-rheumatoid-arthritis-angiogenesis-identified/>
11. Orthopedics this week <http://ryortho.com/breaking/ra-molecules-involved-in-angiogenesis-identified/>

**21.** Kim SJ, Chen Z, Chamberlain ND, Volin MV, Volkov S, Gondzalez M, Vila MO, Gravallesse EM, Arami S, Mehta A, Sweiss N, \***Shahrara S**. Ligation of TLR5 promotes myeloid cell infiltration and differentiation to mature osteoclasts in RA patients. Revisions requested by J Immunol.

**22.** Chen Z , Kim S, Volin MV, Vila OM, Swedler W, Arami A, Volkov S, Sweiss S and **Shahrara S\***. Significance of obesity on RA disease onset and remission. Pending review.

### Invited Lectures:

- |      |  |
|------|--|
| 2010 | University of Illinois in Chicago, Rheumatology rounds   |
| 2010 | American College of Rheumatology Research and Education Foundation <i>Within Our Reach</i> meeting in Dallas |
| 2010 | Northwestern University, Feinberg School of Medicine, Pathology and inflammation group                       |
| 2010 | American College of Rheumatology 2010 annual meeting held in Atlanta   |
| 2011 | Department of Microbiology and Immunology lectures in University of Illinois at Chicago                      |
| 2011 | The Institute for Personalized Respiratory Medicine lectures in University of Illinois at Chicago            |
| 2011 | Department of Rheumatology Grand Rounds in University of Illinois at Chicago                                 |
| 2011 | American College of Rheumatology 2011 annual meeting held in Chicago   |
| 2012 | Department of Microbiology and Immunology lectures in University of Illinois at Chicago                      |

|           |  |
|-----------|--|
| 2012      | Department of Rheumatology Grand Rounds in University of Illinois at Chicago                                     |
| 2012-2013 | Moderator for ACR abstract session “Cytokines, Mediators, Cell-cell adhesion, Cell trafficking and Angiogenesis” |
| 2012      | American College of Rheumatology 2012 annual meeting plenary session held in Washington D.C.                     |
| 2013      | EULAR 2013 held in Spain, Chemokines in monocyte endothelial interactions  |
| 2013      | Invited speaker in Pulmonary Hypertension Seminar Series in University of Illinois at Chicago                    |
| 2013      | Lecturing Rheumatology fellows on the role of cytokines in rheumatoid arthritis                                  |
| 2013      | Invited speaker to Rheumatology Grand Rounds at University of Illinois at Chicago                                |
| 2013      | Invited speaker in UICenter Seminar Series in University of Illinois at Chicago                                  |
| 2014      | Invited speaker to Grand Rounds at New York University   |
| 2014      | Invited speaker to Rheumatology Grand Rounds at University of Illinois at Chicago                                |

**Abstracts and/or oral presentations presented at American College of Rheumatology (ACR) 2010, 2011, 2012, 2013 and 2014:**

1. Pickens SR, Volin MV, Mandelin II AM, **Shahrara S**. Local expression of IL-27 ameliorate collagen induced arthritis. Arthritis Rheum 714: S298, 2010. **Oral presentation**
2. Pickens SR, Volin MV, Pope RM, Mandelin II AM, **Shahrara S**. Characterization of CCR7 and its ligands CCL19 and CCL21 in rheumatoid arthritis. Arthritis Rheum 1506: S627, 2010.
3. Pickens SR, Volin MV, Mandelin II AM, Kolls JK, **Shahrara S**. Anti-CXCL5 therapy ameliorates IL-17-induced arthritis. Arthritis Rheum 270: S112, 2010.
4. Pickens SR, Volin MV, Pope RM, Mandelin II AM, **Shahrara S**. Downstream targets of IL-17 in Rheumatoid Arthritis. Arthritis Rheum 23: S9, 2010.
5. Chamberlain ND, Volin MV, **Shahrara S**. CXCL5 an Important IL-17 Mediated Proangiogenic Factor in Rheumatoid Arthritis and Experimental Arthritis Model. Arthritis Rheum 835: S329, 2011. **Oral presentation.**
6. Chamberlain ND, Pickens SR, Pope RM, Volin MV, **Shahrara S**. IL-7 Contributes to Monocyte Migration in Rheumatoid Arthritis. Arthritis Rheum 729: S284, 2011. **Oral presentation.**

7. Pickens SR, Chamberlain ND, Volin MV, Mandelin AM II, **Shahrara S**. CCL21 a Novel Proangiogenic Factor in Rheumatoid Arthritis. Arthritis Rheum 730: S284, 2011. **Oral presentation.**
8. Chamberlain ND, Volin MV, Pope RM, Mandelin AM II, **Shahrara S**. Characterization of TLR7 and TLR8 in Rheumatoid Arthritis. Arthritis Rheum 364: S136, 2011.
9. Chamberlain ND, Volin MV, Pope RM, Mandelin AM II, **Shahrara S**. TLR5; a Novel and Unidentified Inflammatory Mediator in Rheumatoid Arthritis. Arthritis Rheum 365: S137, 2011.
10. Chamberlain ND, Kim SJ, Volin MV, Mehta A, Sweiss N and **Shahrara S**. IL-7; An Important Pro-inflammatory Factor that affects myeloid cell function in RA and CIA. Arthritis Rheum 328:S143, 2012.
11. Chamberlain ND, Volin MV, Vila OM, Swedler W, Arami S, Volkov S and **Shahrara S**. Expression of TLR5 strongly correlates with levels of TNF- $\alpha$  and DAS28 in RA monocytes and ligation of TLR5 induces angiogenesis in RA. Arthritis Rheum 2454: S1033, 2012. **Oral presentation ACR discoveries of 2012 plenary session.**
12. Chamberlain ND, Kim SJ, Volin MV, Swedler W, Vila OM, Volkov S and **Shahrara S**. TLR7 Ligation Contributes to Monocyte Migration in Rheumatoid Arthritis. Arthritis Rheum 2669:S1132, 2012. **Oral presentation.**
13. Chen Z, Kim SJ, Volin MV, Volkov S, Swedler W, Sweiss N and **Shahrara S**. Characterizing the expression and function of CCL28 and its corresponding receptor CCR10 in the pathogenesis of RA. Arthritis Rheum 933: 2013.
14. Kim SJ, Chen Z, Chamberlain ND, Volin MV, Volkov S, Swedler W, Arami S, Mehta A, Sweiss N and **Shahrara S**. Ligation of TLR5 promotes myeloid cell infiltration and differentiation into mature osteoclasts in RA patients and experimental arthritis. Arthritis Rheum 1152: 2013.
15. Kim SJ, Chen Z, Essani AB, Volin MV, Swedler W, Arami A, Volkov S, Sweiss N and **Shahrara S**. Blockade of TLR5 ligation is a novel strategy for RA therapy. ACR 2014 pending.
16. Chen Z, Kim SJ, Essani AB, Volin MV, Swedler W, Arami S, Volkov S, Fantuzzi G, Sweiss N and **Shahrara S**. The differential impact of obesity on the pathogenesis of RA or preclinical models is contingent on the disease status. ACR 2014 pending.

#### **Abstract for Military Health System Research Symposium (MHSRS) 2014:**

17. Kim SJ, Chen Z, Chamberlain ND, Volin MV, Essani AB and **Shahrara S**. Identifying a novel cascade that modulates IL-17 driven arthritis. Military Health System Research Symposium 2014. Accepted for poster presentation.

## Employment opportunities:

The funding provided to us by DOD enabled me to secure a tenure track position as an Associate Professor of Medicine in University of Illinois at Chicago (UIC). My position started at March 1st of 2011 and the funding provided by DOD was the basis of obtaining this opportunity. I am humbled and grateful for receiving this Investigator Initiated Award from the Department of Defense. Funding from DOD has enabled us to publish 21 papers in high impact factor Journals in short time frame (including an invited Nature Review Rheumatology paper). We are currently preparing 3 more manuscripts which will be submitted before the end of the year. Additionally through the funding available to us from DOD we have presented 6 oral and 8 poster presentations. In endorsement of our productive cutting edge research our most recent findings pertaining to the role of IL-17 in RA angiogenesis was accepted for oral presentation at ACR discoveries of 2012 plenary session. In short as a result of DOD funding, I have established my own independent laboratory and have made great discoveries in finding potential treatment for patients that suffer from RA. I would like to request a no cost extension of our contracted award (PR093477) in order to extend our research performance from 7/1/2014 to 6/30/2015. Accepting our no cost extension for a period of 12 months will ensure that the specific aims of the proposal will be accomplished and we will be able to finalize our studies regarding the significance of IL-17 in RA angiogenesis. Also the funding and support provided by DOD was instrumental for us to obtain funding through NIH R03 and a VA Merit award.

## CONCLUSION

In RA patients percent TH-17 cells correlate with disease activity score suggesting that IL-17 can mediate inflammation at early disease stage and may also be involved in disease progression (16). Therefore to evaluate the role of IL-17 in RA pathogenesis, we examined which IL-17 receptors and signaling pathways are associated with rheumatoid arthritis synovial fluid mediated endothelial migration and tube formation. We show that although HMVECs express both IL-17RA and RC, RA synovial fluid-mediated HMVEC chemotaxis is mediated by binding primarily to IL-17RC. We also report that while IL-17 activates ERK, JNK and PI3K pathways in HMVECs, only inhibition of PI3K reduces IL-17-induced HMVEC chemotaxis and tube formation. We show that IL-17 and VEGF neutralization in RA synovial fluid does not significantly reduce HMVEC migration beyond the effect of one factor alone.

To demonstrate whether there are indirect pathways associated with IL-17 induced angiogenesis, we examined potent proangiogenic factors induced by IL-17 in RA synovial tissue as well as in IL-17 experimental arthritis model. We found that expression of CXCL1 and CXCL5 but not VEGF was highly elevated by IL-17 in RA synovial tissue explants and animal models of RA. To demonstrate the pathologic role of CXCL1 and CXCL5 in IL-17 mediated arthritis, neutralizing antibodies to each chemokine were employed. We found that arthritis severity and vascularization were significantly reduced in the anti-CXCL5 treatment group. In contrast, anti-CXCL1 treatment had no effect on IL-17 mediated disease activity or neovascularization, while being capable of inhibiting CXCL1 mediated endothelial chemotaxis *in vitro*. The combination of anti-CXCL1 and anti-CXCL5 was not more effective than anti-CXCL5 treatment alone. We next demonstrated that ligation to CXCR2 facilitates CXCL1 and CXCL5 induced endothelial migration although downstream signaling pathways are differentially regulated by these chemokines. We show that while CXCL1 can induce endothelial migration through activation of PI3K pathway, this process was mediated by CXCL5 through NF- $\kappa$ B signaling. Since both CXCL1 and IL-17 can mediate angiogenesis through the same pathway, blocking of CXCL1 is ineffective in this process whereas suppression of CXCL5 can effectively reduce NF- $\kappa$ B mediated angiogenesis. In conclusion, these observations suggest that IL-17 mediated joint vascularization may be in part due to CXCL5 induction.

Production of IL-17 from joint TH-17 cells can strongly contribute to RA angiogenesis (14) through a mechanism that is in part due to induction of VEGF from RA ST fibroblasts (17, 18). We document that CCL21 is expressed from endothelial cells activated by IL-17 (19) and neutralization of CCL21 markedly reduces IL-17 mediated VEGF transcription from the RA ST. Like IL-17, CCL21 is also capable of enhancing production of VEGF from RA ST fibroblasts and can further synergize with VEGF in facilitating endothelial chemotaxis. Hence CCL21 may be the unidentified connecting factor between the IL-17 and VEGF cascades. Therapeutic targeting of VEGF and VEGFR has led to disappointing results regarding drug toxicity and lack of efficacy in patients with advanced tumor growth (20, 21) therefore RA patients were not treated with anti-VEGF or anti-VEGFR therapies. However, since we demonstrate that CCL21 induced by IL-17 can modulate VEGF expression in RA ST, targeting CCL21 may disconnect the link between IL-17 and VEGF cascade and therefore more efficiently suppress RA neovascularization.

We show for the first time that expression of CCL28 and CCR10 is markedly higher in RA and OA ST lining macrophages and sublining endothelial cells compared to NL ST. We found that expression of CCL28 in RA myeloid cells is modulated by IL-17. We uncovered that CCL28 strongly attracts endothelial cells at the physiological concentration available in RA SF. Last, we document that knockdown of endothelial CCR10 significantly reduces CCL28 mediated endothelial migration and capillary formation through an ERK dependent mechanism.



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## APPENDICES

1. Our paper published in J Immunol, 2010, 184:3233-3241, supports our finding presented in this progress report.
2. Pickens SR, Chamberlain ND, Volin MV, Mandelin AM 2nd, Agrawal H, Matsui M, Yoshimoto T, **\*Shahrara S**. Local expression of IL-27 ameliorate collagen induced arthritis. Arthritis Rheum 2011, 63:2289-98. **Supports the role of IL-7 and TH-17 in RA angiogenesis.**
3. Pickens SR, Chamberlain ND, Volin MV, Gonzalez M, Pope RM, Mandelin AM II, Kolls JK, **\*Shahrara S**. Anti-CXCL5 therapy ameliorates IL-17-induced arthritis by decreasing joint vascularization. Angiogenesis. 2011,14:443-455. **Supports our progress report.**
4. Chamberlain ND, Vila OM, Volin MV, Volkov S, Pope RM, Swedler W, Mandelin AM 2nd, **\*Shahrara S**. TLR5, a Novel and Unidentified Inflammatory Mediator in Rheumatoid Arthritis that Correlates with Disease Activity Score and Joint TNF- $\alpha$  Levels. J Immunol. 189: 475-83, 2012.
5. Pickens SR, Chamberlain ND, Volin MV, Pope RM, Talarico NE, Mandelin AM II, **\*Shahrara S**. Role of the CCL21 and CCR7 pathway in rheumatoid arthritis angiogenesis. Arthritis Rheum. 2012, 64: 2471-2481. **Supports the role of CCL21 in IL-17 mediated angiogenesis**
6. Chen Z , Kim S, Essani AB, Volin MV, Vila OM, Swedler W, Arami A, Volkov S, Sweiss S and **Shahrara S\***. Characterizing the expression and function of CCL28 and its corresponding receptor CCR10 in RA pathogenesis. Annals Rheum Dis, 2014 In press. **Supports the role of CCL28 in IL-17 mediated angiogenesis.**

# IL-17 Contributes to Angiogenesis in Rheumatoid Arthritis

Sarah R. Pickens,\* Michael V. Volin,<sup>†</sup> Arthur M. Mandelin, II,\* Jay K. Kolls,<sup>‡</sup>  
Richard M. Pope,\*<sup>§</sup> and Shiva Shahrara\*

Angiogenesis is an early and a critical event in the pathogenesis of rheumatoid arthritis (RA). Neovascularization is dependent on endothelial cell activation, migration and proliferation, and inhibition of angiogenesis may provide a novel therapeutic approach in RA. In this study, we document a novel role of IL-17 in mediating angiogenesis. Local expression of IL-17 in mouse ankles increases vascularity. We further demonstrate that IL-17 is angiogenic by showing its ability to promote blood vessel growth in Matrigel plugs *in vivo*. Additionally, IL-17, in concentrations present in the RA joint, induces human lung microvascular endothelial cell (HMVEC) migration mediated through the PI3K/AKT1 pathway. Furthermore, suppression of the PI3K pathway markedly reduces IL-17–induced tube formation. We also show that both IL-17–induced HMVEC chemotaxis and tube formation are mediated primarily through IL-17 receptor C. Neutralization of either IL-17 in RA synovial fluids or IL-17 receptor C on HMVECs significantly reduces the induction of HMVEC migration by RA synovial fluid. Finally, RA synovial fluid immunoneutralized with anti-IL-17 and antivascular endothelial growth factor does not reduce HMVEC migration beyond the effect detected by immunodepleting each factor alone. These observations identify a novel function for IL-17 as an angiogenic mediator in RA, supporting IL-17 as a therapeutic target in RA. *The Journal of Immunology*, 2010, 184: 3233–3241.

Helper-17 cells are a newly discovered CD4<sup>+</sup> helper T cell subset that produce IL-17A (also known as IL-17). IL-17 binds to transmembrane receptors, which exist as IL-17 receptor A (IL-17RA) homodimers or IL-17RA/IL-17 receptor C (IL-17RC) heterodimers (1). IL-17 RA and RC are widely expressed in endothelial cells, B and T cells, fibroblasts, and monocytes (2, 3). TGF- $\beta$ , IL-6, IL-1 $\beta$ , and IL-21 have been shown to drive the differentiation of Th-17 cells (4–6), although variation between humans and mice has been described previously (2, 6–8). In humans, IL-1 $\beta$  is the most effective inducer of Th-17 cell differentiation, although this process is enhanced by IL-6 and IL-23 (6).

IL-17 has been shown to have a profound effect in experimental models of arthritis. Local expression of IL-17 in mouse joints results in acute inflammation and cartilage proteoglycan depletion (9). The incidence and severity of collagen-induced arthritis was markedly attenuated in IL-17–deficient mice (10). IL-17 also plays an important role downstream of IL-1 signaling and in response to TLR4 ligands (11, 12). Spontaneous IL-1 receptor antagonist knockout mice develop a polyarthritis, which is completely suppressed when

these mice are crossed with IL-17–deficient mice (13), suggesting that Th-17 cell differentiation may be the reason for the arthritis.

IL-17 is found in rheumatoid arthritis (RA) synovial tissue and fluid, and the percentage of Th-17 cells is significantly higher in RA synovial fluid compared with RA or normal peripheral blood (3, 14). IL-17 may play an important role in the pathogenesis of RA by inducing the production of proinflammatory cytokines and chemokines from RA synovial tissue fibroblasts including IL-6, CXCL8/IL-8, CCL2/MIP-3 $\alpha$ , CXCL1/growth-related oncogene- $\alpha$ , and CXCL2/growth-related oncogene- $\beta$  (15–17). IL-17 is also capable of activating macrophages to expression of IL-1, TNF- $\alpha$ , cyclooxygenase 2, PGE<sub>2</sub>, and matrix metalloproteinase-9 (18–20). We recently demonstrated that IL-17 is directly chemotactic for monocytes (21), and others have shown that IL-17 activates neutrophil recruitment through the stimulation of chemokines such as CXCL1 in rats and CXCL8/IL-8 in humans (22, 23).

Angiogenesis, the development of new capillaries, is involved in leukocyte ingress into the synovium during the development and progression of RA (24, 25). However, the role of IL-17 in angiogenesis is undefined. There are contradictory results regarding the role of IL-17 on tumor development and angiogenesis. In mice, tumors transfected with IL-17 demonstrated markedly less growth compared with control tumors (26). Additionally, tumor growth and lung metastasis were increased in IL-17–deficient mice (27), suggesting that IL-17 inhibits tumor development and neovascularization. Others have shown that IL-17 increases blood vessel development in rat cornea and tumor vascularity in animal models, indicating that IL-17 may be important for angiogenesis (28). However, there are also data to suggest that IL-17 alone is unable to induce angiogenesis but can indirectly mediate human lung microvascular endothelial cell (HMVEC) growth by promoting the mitogenic activity of vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), and hepatocyte growth factor (29, 30). Therefore, on the basis of the existing data, it is unclear whether IL-17 can promote angiogenesis in RA and whether IL-17 acts directly on endothelial cells through

\*Division of Rheumatology, Department of Medicine, Feinberg School of Medicine, Northwestern University; <sup>†</sup>Jesse Brown Veterans Affairs Hospitals, Chicago Healthcare System, Chicago, IL 60611; <sup>‡</sup>Department of Microbiology and Immunology, Chicago College of Osteopathic Medicine, Midwestern University, Downers Grove, IL 60515; and <sup>§</sup>Department of Genetics, Louisiana State University Health Sciences Center, New Orleans, LA 70112

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Address correspondence and reprint requests to Dr. Shiva Shahrara, Division of Rheumatology, Department of Medicine, Northwestern University Feinberg School of Medicine, McGaw Pavilion, 240 East Huron Street, Suite M300, Chicago, IL 60611. E-mail address: s-shahrara@northwestern.edu

Abbreviations used in this paper: Ad, adenovirus; bFGF, basic fibroblast growth factor; EBM, endothelial basal medium; Endo, endothelial cell; FGF, fibroblast growth factor; HEK, human embryonic kidney; HMVEC, human lung microvascular endothelial cell; IL-17RA, IL-17 receptor A; IL-17RC, IL-17 receptor C; p, phospho; RA, rheumatoid arthritis; VEGF, vascular endothelial growth factor.

binding to its receptors or indirectly by inducing proangiogenic factors from cells present in the RA synovium.

In the current study, we have therefore evaluated the role of IL-17 in HMVEC migration and tube formation as well as blood vessel development. Our results demonstrate that local expression of IL-17 increases vascularity in mouse ankle joints. Using a Matrigel plug assay, we demonstrate that IL-17 enhances blood vessel formation *in vivo*. Although HMVECs express both IL-17RA and RC, IL-17-induced HMVEC chemotaxis and tube formation are mediated by binding primarily to IL-17RC and activation of the PI3K pathway. We report that RA synovial fluid-mediated endothelial migration is significantly reduced by IL-17 and/or IL-17RC neutralization. Finally, we show that IL-17 and VEGF neutralization in RA synovial fluid does not significantly reduce HMVEC migration beyond the effect of one factor alone. Hence, therapy directed against IL-17 may reduce leukocyte migration by inhibiting angiogenesis in RA.

## Materials and Methods

### *Study protocol for local expression of IL-17 in mouse ankle joints*

The animal studies were approved by the Northwestern University Institutional Review Board. Adenovirus (Ad) constructed as reported previously to contain an IL-17 payload was provided by J. K. Kolls (Louisiana State University Health Science Center, New Orleans, LA) (31). Four- to six-week-old C57BL/6 mice were injected intra-articularly with  $10^7$  PFU Ad-IL-17 or Ad-CMV control. Ankle circumference and articular index score were determined on days 4 and 10 post-Ad-IL-17 injection and compared with the control group (data not shown). Ankles were harvested on day 10 post-Ad-IL-17 injection for histological studies. Levels of IL-17 were quantified by ELISA on days 4 and 10 from ankles treated with Ad-IL-17 or Ad-CMV control.

### *Abs and immunohistochemistry*

Mouse ankles were decalcified, formalin fixed and paraffin embedded, and sectioned in the pathology core facility of Northwestern University. Mouse ankles were immunoperoxidase stained using Vector Elite ABC Kits (Vector Laboratories, Burlingame, CA), with diaminobenzidine (Vector Laboratories) as a chromogen. Slides were deparaffinized in xylene for 20 min at room temperature, followed by rehydration by transfer through graded alcohols. Abs were unmasked by first incubating slides in boiling citrate buffer for 15 min, followed by type II trypsin digestion for 30 min at 37°C. Endogenous peroxidase activity was blocked by incubation with 3% H<sub>2</sub>O<sub>2</sub> for 5 min. Nonspecific binding of avidin and biotin was blocked using an avidin/biotin blocking kit (Vector Laboratories). Nonspecific binding of Abs to the tissues was blocked by pretreatment of tissues with diluted normal goat serum. Tissues were incubated with rabbit polyclonal Ab to von Willebrand factor (1/100 dilution; Serotec, Oxford, U.K.) or a rabbit IgG control Ab (Beckman Coulter, Fullerton, CA). Slides were counterstained with Harris hematoxylin and treated with lithium carbonate for bluing. Each slide was evaluated by a blinded observer (32–35) (M.V.V.). Tissue sections were assigned a vascular score representing the number of blood vessels in each section. A semiquantitative score of 1 represented a tissue with few blood vessels, whereas a score of 4 represented a highly vascularized tissue. Scored data were pooled, and the mean  $\pm$  SEM was calculated in each data group ( $n = 5$ ).

### *Matrigel plug assay in vivo*

To examine the effect of IL-17 on angiogenesis *in vivo*, we used a Matrigel plug assay. Four- to six-week-old C57BL/6 mice were injected s.c. in the dorsal area with 500  $\mu$ l Matrigel. Matrigel plus PBS served as negative control, Matrigel containing bFGF (20 ng/ml) served as positive control, and experimental conditions included Matrigel with mouse recombinant IL-17 (2  $\mu$ g). After 10 d, mice were sacrificed, Matrigel plugs were carefully dissected out, surrounding connective tissue was removed, and plugs were analyzed for vascularity by hemoglobin measurement or by histology. For hemoglobin measurement, plugs were weighed by placing them into 1 ml preweighed double-distilled H<sub>2</sub>O and then homogenized for 5–10 min on ice and spun. Using methemoglobin, serial dilutions were prepared to generate a standard curve from 70 to 1.1 g/dl (36, 37). Fifty microliters of supernatant or standard was added to a 96-well plate in duplicate, and 50  $\mu$ l tetramethylbenzidine was added to each sample. The plate was allowed to develop at room temperature for 15–20 min with gentle shaking, and the reaction was terminated with 150  $\mu$ l 2 N H<sub>2</sub>SO<sub>4</sub> for 3–5 min. Absorbance was read with an ELISA

plate reader at 450 nm. To calculate hemoglobin concentrations, the values (grams per deciliter) were normalized to the weights of the plugs (grams) (36, 37). On day 10, IL-17 concentrations were quantified in Matrigel plugs harvested from the IL-17-treated group using ELISA.

For histology, plugs were embedded in paraffin and sectioned in the pathology core facility of Northwestern University. Histology slides from different groups were examined by H&E and Masson's trichrome staining (37, 38) and scored by a blinded observer (A.M.M.) based on a scale of 0–4, where 0 = no tubules, 1 = tubules only, 2 = tubules containing RBCs, 3 = tubules with multilayer wall, and 4 = tubules with multilayer walls with connective tissues surrounding them.

### *HMVEC chemotaxis*

To examine chemotaxis, HMVECs were incubated in endothelial basal medium (EBM) with 0% FBS and no growth factors for 2 h before use. HMVECs ( $1.25 \times 10^4$  cells/25  $\mu$ l EBM with 0.1% FBS) were then placed in the bottom wells of a 48-well Boyden chemotaxis chamber (NeuroProbe, Cabin John, MD) with gelatin-coated polycarbonate membranes (8- $\mu$ m pore size; Nucleopore, Pleasant, CA) (37, 39). The chambers were inverted and incubated at 37°C for 2 h, allowing endothelial cell attachment to the membrane. The chamber was reinverted, and PBS, positive control VEGF (10 ng/ml; R&D Systems, Minneapolis, MN), or IL-17 at varying concentrations from 0.001 to 100 ng/ml (R&D Systems) was added to the upper wells, and the chamber was further incubated for 2 h at 37°C. The membranes were then removed, fixed, and stained with the Protocol HEMA 3 stain set (122-911; Fisher Scientific, Waltham, MA). Readings represent the number of cells migrating through the membrane (the average of three high-power  $\times 40$  fields/well, averaged for each triplicate of wells). To test specificity of IL-17-induced HMVEC migration, HMVEC chemotaxis was examined with heat-inactivated IL-17 (10 and 50 ng/ml incubated at 100°C for 15 min) or IL-17 neutralized by anti-IL-17 Ab or IgG control (10  $\mu$ g/ml for 1 h in 37°C) (R&D Systems) (21). To examine for chemokinesis, a series of checkerboard experiments were performed by placing increasing concentrations of IL-17 (0, 0.01, 1, and 10 ng/ml) together with HMVECs in the bottom wells with different concentrations of IL-17 in the top wells. To determine which IL-17 receptors are important for IL-17 HMVEC migration, Abs to IL-17RA (R&D MAB177; 10  $\mu$ g/ml), IL-17RC (R&D AF2269; 10  $\mu$ g/ml), both Abs, or IgG control were added to HMVECs, incubated for 1 h at 37°C, and further incubated for 2 h in the Boyden chamber with IL-17 (50 ng/ml). To define which signaling pathway(s) mediated IL-17-induced HMVEC chemotaxis, HMVECs were incubated with inhibitors to PI3K (LY294002; 1 and 5  $\mu$ M), ERK (PD98059; 1 and 5  $\mu$ M), JNK (SP600125; 1 and 5  $\mu$ M), or DMSO for 2 h in the Boyden chamber with IL-17 (50 ng/ml). HMVEC chemotaxis induced by RA synovial fluids was examined following 1-h incubation (37°C) of fluids with control IgG or neutralizing anti-IL-17 Ab (10  $\mu$ g/ml). The fluids were diluted 1/20 before addition to the top wells (21). To examine whether IL-17 receptors are involved in RA synovial fluid-induced HMVEC chemotaxis, HMVECs were incubated with Abs to IL-17RA and RC (10  $\mu$ g/ml) or both Abs, as well as IgG control (1 h at 37°C), before adding the RA synovial fluid to the top wells (21). HMVEC chemotaxis was performed to examine the synergistic effect of IL-17 and/or VEGF in RA synovial fluid induced HMVEC migration. Chemotaxis induced by RA synovial fluids was examined following incubation of fluids (diluted 1/20) ( $n = 8$  fluids) with control IgG, anti-IL-17, anti-VEGF, or both Abs (10  $\mu$ g/ml) for 1 h prior to performing the assay.

### *Quantification of IL-17RA and RC expression on HMVECs*

Skin and lung HMVECs, HUVECs, and human embryonic kidney (HEK) 293 were cultured in EGM-2, EGM, and 10% DMEM, respectively. Total cellular RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) from all different cell types. Subsequently, reverse transcription and real-time RT-PCR were performed to determine IL-17RA and IL-17RC expression level as described previously (14, 21, 40). Relative gene expression was determined by the  $\Delta\Delta C_t$  method, and results were expressed as fold increase above levels detected in HEK 293 cells.

### *Characterization of IL-17 signaling pathways in HMVECs*

HMVECs (passages 3–8) were grown to 80% confluence in EGM-2 MV bullet kit (Lonza, Walkersville, MD). Cells were then switched to EGM-MV (EBM plus SingleQuots of growth supplements) for 2 d and were incubated in EBM with 0% FBS for 2 h prior to treatment. Cells were then untreated or treated with IL-17 (50 ng/ml) for 15–75 min. Cell lysates were examined by Western blot analysis, as described previously (21, 40, 41). Blots were probed with phospho (p)-AKT, pERK, and pJNK (1/1000 dilution; Cell Signaling Technology, Beverly, MA) overnight and after stripping were probed with AKT, ERK, and JNK (1/3000 dilution; Cell Signaling Technology) overnight.



### HMVEC tube formation assay

To perform the Matrigel tube formation assay, BD Matrigel Matrix (BD BioSciences, Bedford, MA; 50  $\mu$ l) was polymerized for 30 min at 37°C in a 96-well plate. To determine which IL-17 receptors play a role in IL-17–induced HMVEC tube formation, trypsinized HMVECs were resuspended ( $4 \times 10^5$  cells/ml) in EBM without growth factors with 2% FBS and incubated with Abs to IL-17RA, IL-17RC, both IL-17RA and RC or IgG for 45 min at 37°C. Cells were then added to polymerized Matrigel, IL-17 (50 ng/ml) was added to the wells, and the plate was incubated for 16 h at 37°C. To examine which signaling pathways contribute to IL-17–mediated HMVEC tube formation, cells were incubated with inhibitors to PI3K (LY294002; 1 and 5  $\mu$ M), ERK (PD98059; 1 and 5  $\mu$ M), JNK (SP600125; 1 and 5  $\mu$ M), or DMSO for 45 min at 37°C prior to adding to polymerized Matrigel. As above, IL-17 (50 ng/ml) was then added to the wells, and the plate was incubated for 16 h at 37°C. Each condition was performed in triplicate, fibroblast growth factor (FGF) (20 ng/ml) was used as a positive control, and PBS was used as a negative control. Following incubation, culture medium was removed, and cells were washed with HBSS. Thereafter, tube formation was quantified using calcein-AM fluorescent dye (BD BioSciences) diluted with HBSS and DMSO, according to the manufacturer's instructions, for 30 min. Subsequently, the plates were washed with HBSS, and the number of branch points/tubes was quantified as described previously (36, 37).

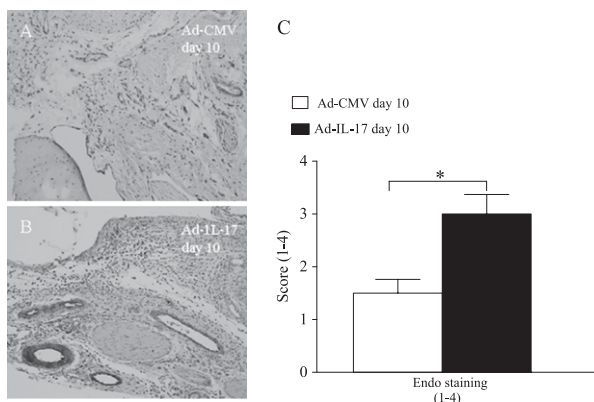
### Statistical analysis

The data were analyzed using two-tailed Student *t* tests for paired and unpaired samples. Values of  $p < 0.05$  were considered significant.

## Results

### Local expression of IL-17 in mouse ankles induces joint inflammation and vascularity

Local expression of IL-17 using an adenoviral vector ( $10^7$  PFU) resulted in increased inflammation, synovial lining thickness, and bone erosion in the ankles of C57/BL6 mice, compared with Ad-CMV–infected controls ( $10^7$  PFU) (data not shown). The Ad-IL-17–treated group demonstrated significantly greater ankle circumference (data not shown) on days 4 and 10 postinjection compared with the control group. Von Willebrand factor staining of ankles harvested from day 10 postinjection demonstrated that Ad-IL-17–treated mice have significantly higher endothelial staining compared with the control group (Fig. 1). The concentration of joint IL-17 in the IL-17–induced arthritis model was 1200 and 400 pg/mg compared with 47 and 31 pg/mg detected in the Ad-CMV



**FIGURE 1.** Local expression of IL-17 increases vascularity in mouse ankles. Ad-IL-17 or Ad-CMV control was injected intra-articularly into the ankle joints of 4–6-wk-old C57BL/6 mice. Ankles from day 10 post-Ad injection were harvested, paraffin embedded and decalcified. Ankles were stained with Von Willebrand factor for identifying endothelial cells (Endo) and scored on a 1–4 scale. Control ankles (A) had significantly lower endothelial staining compared with ankles locally expressing IL-17 (original magnification  $\times 200$ ) (B). C, Quantification of each of the parameters in the Ad-CMV control and Ad-IL-17 groups. Values demonstrate mean  $\pm$  SE;  $n = 5$ . \* $p < 0.05$ .

control group on days 4 and 10 post-Ad injection. These results suggest that IL-17 may be important for angiogenesis in vivo.

### IL-17 induces angiogenesis in vivo in Matrigel plugs

The role of IL-17 on angiogenesis in vivo was assessed by determining its effect on blood vessel formation in Matrigel plugs in mice by using hemoglobin quantification, as well as Masson's trichrome staining. The hemoglobin content of the IL-17–treated group was 10 times greater ( $p < 0.05$ ) than the PBS control (Fig. 2A). Matrigel blood vessel formation was also examined histologically by using H&E (Fig. 2C, 2E, 2G) and Masson's trichrome staining (Fig. 2D, 2F, 2H). The histological analysis demonstrated that IL-17 markedly enhances ( $p < 0.05$ ) blood vessel growth compared with the control group (Fig. 2B). The levels of IL-17 detected in Matrigel plugs harvested on day 10 were  $198 \pm 35$  pg/ml, which is within the range detected in IL-17–induced arthritis model as well as in the RA synovial fluid (mean was 233 pg/ml). These results support the role of IL-17 in angiogenesis in vivo.

### IL-17 induces endothelial (HMVEC) migration

Experiments were performed to determine whether IL-17 is directly chemotactic for endothelial cells. For this purpose, chemotaxis was performed in a Boyden chamber with varying concentrations of IL-17, as well as positive (VEGF; 10 ng/ml) and negative (PBS) controls. IL-17 was chemotactic for HMVECs at concentrations ranging from 0.01 ng/ml ( $p < 0.05$ ) to 100 ng/ml ( $p < 0.05$ ) ( $n = 5$ ) (Fig. 3A). The mean concentration of IL-17 in the 30 RA synovial fluids analyzed was  $233 \pm 64$  pg/ml (21), a concentration that was highly chemotactic for HMVEC migration. IL-17's endotoxin levels were quantified by using *Limulus* amoebocyte cell lysate assay. At a concentration of IL-17 10-fold higher than that used in the HMVEC chemotaxis assay (500 ng/ml), the level of endotoxin was below the detection limit for this assay ( $< 0.01$  ng/ml LPS) (data not shown), suggesting that our results are specifically due to IL-17 and not endotoxin contamination. Supporting this interpretation, heat inactivation of IL-17 or incubation of IL-17 (50 ng/ml) with neutralizing Ab to IL-17 suppressed HMVEC migration (Fig. 3B).

We next determined the effect of IL-17 on chemokinesis. In the absence of IL-17 in the lower chamber, IL-17 in the upper chamber was chemotactic for HMVECs. When equivalent or higher concentrations of IL-17 were present in the lower compartment, no enhanced migration of HMVECs occurred (Fig. 3C). Taken together, our results suggest that IL-17 is chemotactic, not chemokinetic, for endothelial cells.

### IL-17 receptors are involved in IL-17–induced HMVEC chemotaxis and HMVEC tube formation

Our results demonstrate that skin and lung HMVECs as well as HUVECs express significantly higher levels of IL-17RA and IL-17RC compared with HEK 293 cells (Fig. 4A, 4B). These experiments were performed to determine which IL-17 receptor is involved in HMVEC chemotaxis and tube formation. Although some reduction of HMVEC chemotaxis was noted with an anti-IL-17RA Ab, it was not significant. Inhibition of chemotaxis was significant when IL-17RC (40%) or both receptors (47%) were neutralized (Fig. 5A). IL-17 also induced HMVEC tube formation in Matrigel. Using concentrations of IL-17 ranging from 0.001 to 100 ng/ml, the optimal concentration of IL-17 to induce HMVEC tube formation was 50 ng/ml ( $p < 0.05$ ) (data not shown). Consistent with the HMVEC chemotaxis data, neutralization of IL-17RC (30%) or both IL-17 receptors (45%) on HMVECs significantly decreased ( $p < 0.05$ ) IL-17–mediated HMVEC tube formation (Fig. 5B–H). Blockade of IL-17RA alone was ineffective in this process. Collectively, our data suggest that IL-17RC plays a more important role

in IL-17-mediated HMVEC chemotaxis and tube formation compared with IL-17RA.

*IL-17 activates ERK, JNK, and PI3K pathways in HMVECs; however only inhibition of PI3K reduces IL-17-induced HMVEC chemotaxis and tube formation*

To determine which signaling pathways in HMVECs are activated by IL-17, phosphorylation of the MAPK and AKT pathways was determined by Western blot analysis. Our data demonstrate that IL-17 phosphorylates ERK, JNK, and AKT1 as early as 15 min. Although the activation of ERK and JNK is gradually reduced at 75 and 65 min (Fig. 6B, 6C), respectively, AKT1 is still strongly phosphorylated at 75min (Fig. 6A). The p38 pathway was not activated by IL-17 in HMVECs (data not shown). To determine which signaling pathways mediate HMVEC migration, chemical inhibitors at concentrations of 1 and 5  $\mu$ M were used, whereas 10  $\mu$ M was toxic and resulted in cell death, as determined by trypan blue staining (data not shown). Inhibition of ERK and JNK was ineffective in suppressing IL-17-induced HMVEC chemotaxis, whereas inhibition of PI3K reduced ( $p < 0.05$ ) chemotaxis starting at 1  $\mu$ M (Fig. 6D). Similarly, although inhibition of PI3K (starting at 1  $\mu$ M) reduced IL-17-mediated tube formation by 30–40% ( $p < 0.05$ ) (Fig. 7), suppression of ERK and JNK had no effect on this process. These results suggest that IL-17-induced HMVEC chemotaxis and tube formation are mediated through the PI3K/AKT1 pathway.

*IL-17 and its receptor are involved in RA synovial fluid-mediated HMVEC chemotaxis*

Next, we asked whether the IL-17 identified in human RA synovial fluid is chemotactic for HMVECs. In these experiments, we demonstrated that human RA synovial fluid is chemotactic for HMVECs, similar to positive control VEGF (Fig. 8A). Furthermore, human RA synovial fluid immunodepleted with anti-IL-17 significantly reduced HMVEC chemotaxis ( $p < 0.05$ ), compared with control IgG-treated fluids. Neutralization of IL-17RA and RC on HMVECs showed that only IL-17RC was effective in suppressing human RA synovial fluid-mediated HMVEC migration (Fig. 8B). These results suggest that IL-17 and its receptor IL-17RC may play an important role in angiogenesis in RA. Fur-

thermore, we demonstrate that immunodepletion of IL-17 and VEGF in RA synovial fluids does not have an additive or synergistic effect in reduction of HMVEC migration beyond the effect noted with neutralization of one factor alone (Fig. 8C), suggesting that both IL-17 and VEGF may be mediating HMVEC migration through the same signaling pathway.

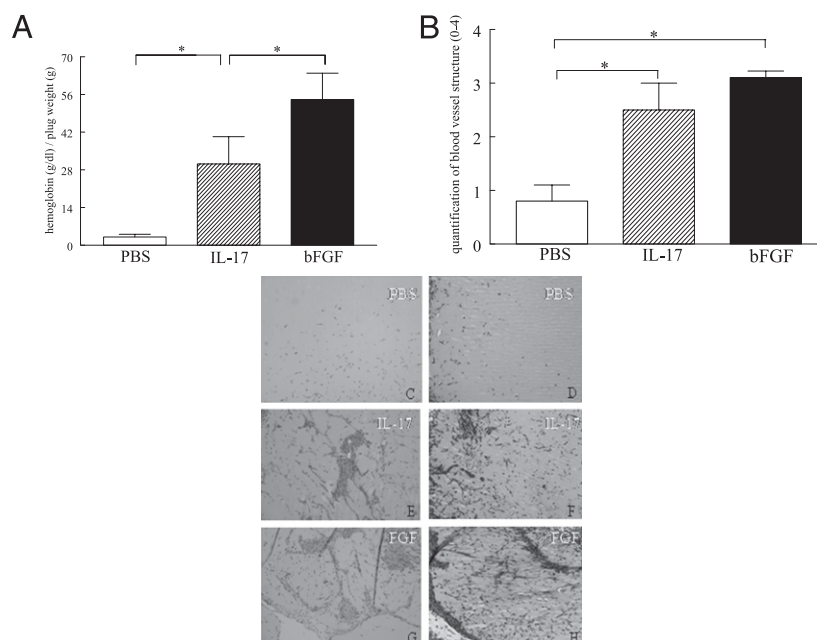
## Discussion

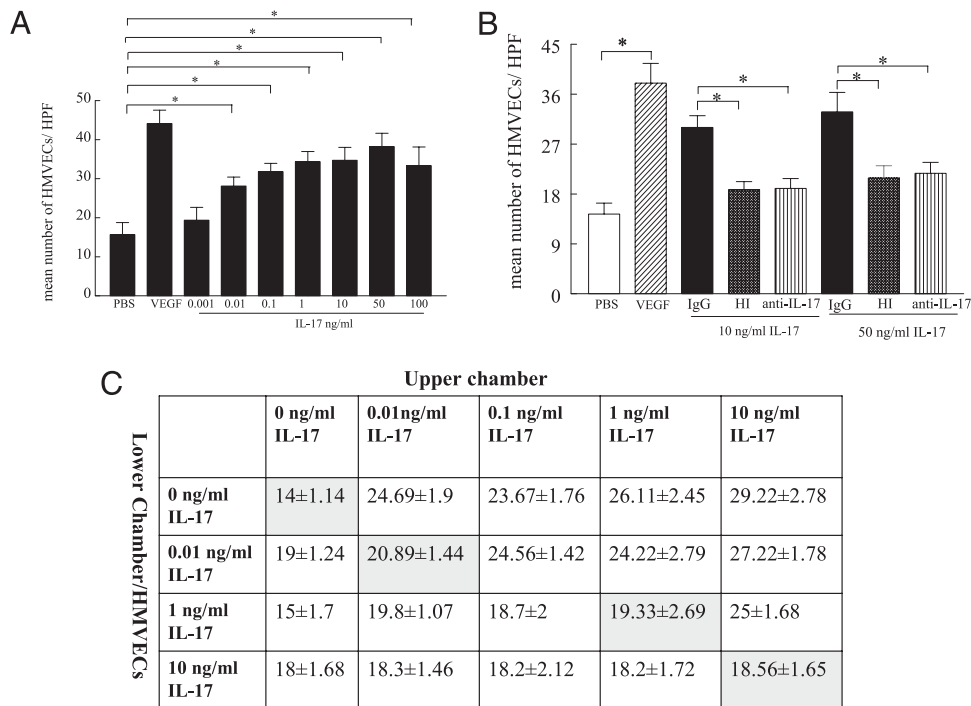
In this study, we show that IL-17 increases vascularity in experimental arthritis and induces blood vessel development in Matrigel plugs *in vivo*. Therefore, studies were performed to determine whether IL-17 might directly mediate angiogenesis and whether IL-17 contributes to neovascularization in RA. Our data demonstrate that IL-17 induces HMVEC chemotaxis at concentrations present in human RA synovial fluid. This effect is directly mediated by IL-17, because heat inactivation and neutralizing Abs to IL-17 and/or IL-17 receptors abrogate IL-17-induced HMVEC chemotaxis. We further demonstrate that IL-17-induced HMVEC chemotaxis and tube formation are mediated primarily through ligation to IL-17RC on HMVECs and activation of PI3K. We show that human RA synovial fluid-mediated HMVEC chemotaxis is markedly reduced by neutralization of IL-17 in the synovial fluids or blocking of IL-17RC on HMVECs. Last, we demonstrate that IL-17 and VEGF-immunodepleted RA synovial fluid does not reduce HMVEC chemotaxis any further than neutralization of each factor by itself.

In this paper, we confirm the results of others demonstrating that the local expression of IL-17 in mouse ankle joints induces arthritis (9). Histological analysis of mice receiving intra-articular injections of IL-17 demonstrated that IL-17 plays an important role in joint neutrophil migration (42). We demonstrate that IL-17-induced arthritis is associated with increased vascularity. Others have shown that IL-17 can promote tumor growth by upregulating proangiogenic factors such as VEGF and matrix metalloproteinase-9 from tumor cells, suggesting that IL-17 is indirectly associated with angiogenesis (43). We have also shown that forced ectopic expression of IL-17 induces expression of proangiogenic CXCL1 (ELR<sup>+</sup>) chemokines in mouse ankles (S. Pickens and S. Shahrara, unpublished data).

On the basis of our results from the IL-17-induced arthritis model, we hypothesized that IL-17 may be important for angiogenesis in RA. Because there is some evidence demonstrating that

**FIGURE 2.** IL-17 enhances blood vessel growth in Matrigel plugs *in vivo*. **A**, IL-17 (2  $\mu$ g) induced angiogenesis in the Matrigel plugs to a significantly greater degree compared with PBS control. Matrigel containing bFGF (20 ng/ml) served as positive control. The values represent the concentration of hemoglobin (grams per deciliter)/plug weight (grams)  $\pm$  SE, with  $n = 10$ . **B**, The histology was quantified on a score of 0–4 scale in Matrigel plugs that contained PBS, IL-17, or bFGF using H&E and Masson's trichrome staining. A representative assay shows H&E (C, E, G) and Masson's trichrome (D, F, H) staining of blood vessels in paraffin sections of Matrigel plugs containing PBS (C, D), IL-17 (E, F), and FGF (G, H) that was histologically scored in B (original magnification  $\times 200$ ). Values demonstrate mean  $\pm$  SE, with  $n = 5$ . \* $p < 0.05$ .





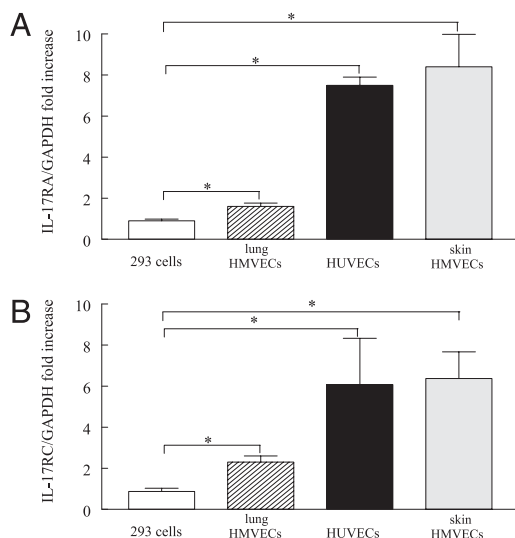
**FIGURE 3.** IL-17 induces HMVEC migration and not chemokinesis. **A**, Dose-response curve of IL-17-induced HMVEC chemotaxis. IL-17 HMVEC chemotaxis was performed in a Boyden chemotaxis chamber with varying concentrations of IL-17. Values demonstrate mean  $\pm$  SE from five different experiments. **B**, IL-17-induced HMVEC chemotaxis was suppressed by heat inactivating IL-17 (both 10 and 50 ng/ml incubated in 100°C for 15 min) or neutralization of IL-17 (10 and 50 ng/ml) by anti-IL-17 Ab but not by IgG control (10  $\mu$ g/ml 1 h in 37°C) for 2 h. Values are the mean  $\pm$  SE from three different experiments.  $*p < 0.05$ . **C**, A series of checkerboard experiments was performed by placing increasing doses of IL-17 together with HMVECs in the lower well in addition to placing different concentrations of IL-17 in the top well of the chemotaxis chamber. The experiment was read at 2 h. Results are representative of three different experiments.

IL-17 alone is unable to induce angiogenesis but can indirectly promote HMVEC chemotaxis by producing proangiogenic factors (29, 30) from RA synovial tissue fibroblasts, we investigated the role of IL-17 on HMVEC migration and tube formation. Our results demonstrate that IL-17 induces HMVEC chemotaxis at concentrations available in the human RA joint, which is mostly

due to its ligation to IL-17RC. Although IL-17RC plays a major role in IL-17-mediated HMVEC chemotaxis and tube formation, neutralization of both receptors is more effective in this process compared with IL-17RC alone. Like monocytes, HMVECs express both IL-17RA and IL-17RC (21). However, in contrast to HMVEC chemotaxis, IL-17-mediated monocyte migration is induced through both IL-17RA and RC (21). Interestingly, a novel IL-17 receptor-like protein has been identified in HUVECs that interacts with FGF1 and inhibits activation of the ERK pathway and production of FGF, indicating that various IL-17 receptors may modulate angiogenesis differently (44).

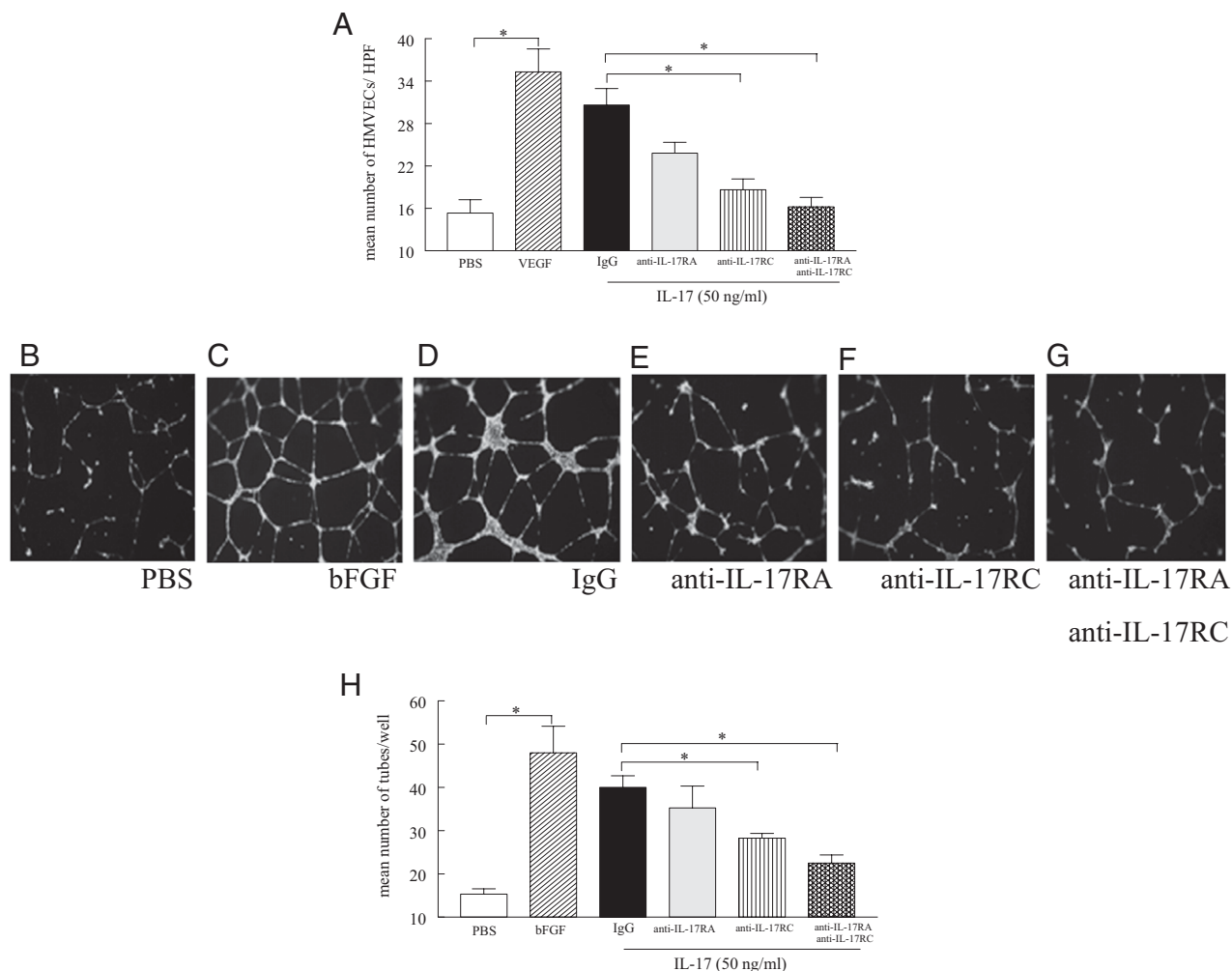
Next, experiments were performed to investigate signaling pathways that were associated with IL-17-induced HMVEC chemotaxis and tube formation. Inhibition of the IL-17-activated pathways in HMVECs demonstrated that only activation of PI3K significantly reduces IL-17-mediated chemotaxis and tube formation, and suppression of ERK and JNK pathways was ineffective in this process. Consistently, CCL2/MCP-1-mediated endothelial chemotaxis is through activation of PI3K, as well as the ERK pathway (45). Furthermore, others have shown that PI3K signaling plays an important role in regulation of VEGF production as well as VEGF-mediated endothelial migration (46, 47), suggesting that PI3K is involved in the mediation of angiogenesis by various proinflammatory factors. A recently published article shows that increased expression of IL-17 in IFN $\gamma^{-/-}$  mice mediates tumor growth and angiogenesis through STAT3 phosphorylation (43). In contrast to IL-17, which promotes angiogenesis, IL-17F inhibits HUVEC tube formation, indicating that different IL-17 isoforms may modulate angiogenesis differently (48).

A recent publication demonstrated that tumor growth in s.c. and lung metastases are enhanced in IL-17 $^{-/-}$  mice compared with the wild-type controls, suggesting that IL-17 may suppress tumor



**FIGURE 4.** HMVECs express IL-17RA (**A**) and RC (**B**). Skin and lung HMVECs, HUVECs, and HEK 293 were cultured and real-time RT-PCR was performed to determine IL-17RA and IL-17RC expression level. The relative gene expression levels were normalized by GAPDH and determined by the  $\Delta\Delta C_t$  method, and results were expressed as fold increase above levels detected in HEK 293 cells. Values are the mean  $\pm$  SE,  $n = 3-6$ .  $*p < 0.05$ .

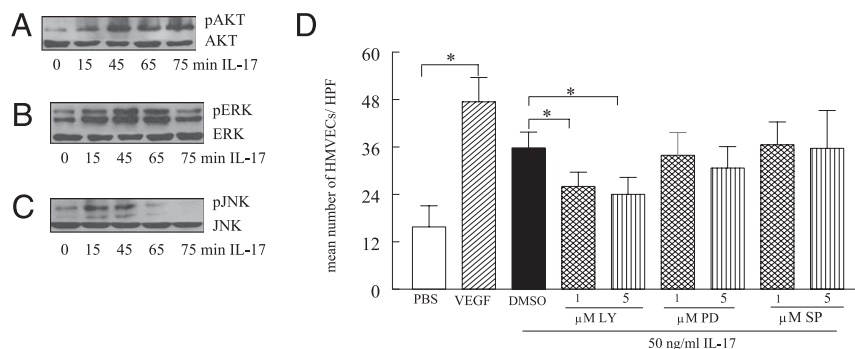




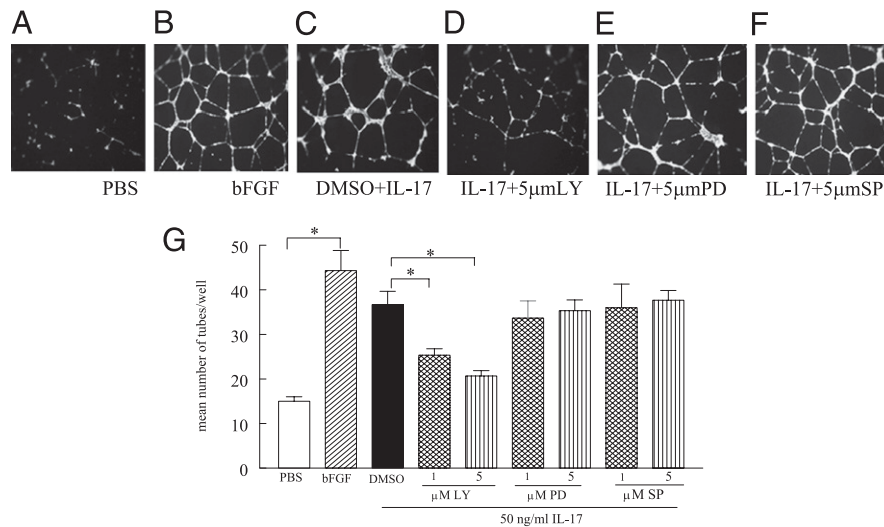
**FIGURE 5.** IL-17-mediated HMVEC chemotaxis and tube formation are regulated through both IL-17RA and IL-17RC. *A*, HMVECs were incubated with mouse anti-human IL-17RA and IL-17 RC Abs (10  $\mu$ g/ml) or control IgG (10  $\mu$ g/ml) for 1 h. Thereafter, HMVEC chemotaxis was performed in response to IL-17 (50 ng/ml) for 2 h. PBS was used as a negative control and VEGF (60 nM) as a positive control. HMVECs were incubated with Abs to IL-17RA, IL-17RC, both IL-17RA and RC, or IgG for 45 min at 37°C. Cells were then added to polymerized Matrigel, IL-17 (50 ng/ml), placed in the wells, and the plate was incubated for 16 h at 37°C (in triplicate). Photomicrographs (original magnification  $\times 100$ ) taken of representative wells treated with PBS (*B*), FGF (20 ng/ml) (*C*), IL-17 (50 ng/ml) plus IgG (*D*), IL-17 (50 ng/ml) plus anti-IL-17RA (10  $\mu$ g/ml) (*E*), IL-17 (50 ng/ml) plus anti-IL-17RC (10  $\mu$ g/ml) (*F*), and IL-17 (50 ng/ml) plus anti-IL-17RA and RC (10  $\mu$ g/ml) (*G*) in which IL-17-induced tube formation is significantly reduced by the neutralization of IL-17RC or both receptors ( $p < 0.05$ ). *H*, Data presented demonstrates mean number of branch points/tubes in each treatment group. Values are the mean  $\pm$  SE,  $n = 3$ . \* $p < 0.05$ .

development (27). Conversely, others have shown that IL-17 markedly increases neovascularization in rat cornea (28) and vascularization in tumors (49), indicating that IL-17 may promote

angiogenesis. However, there is also evidence demonstrating that IL-17 induces production of proangiogenic factors including NO, hepatocyte growth factor, CXCL1/KC, CXCL2/MIP-2, PGE<sub>1</sub>,



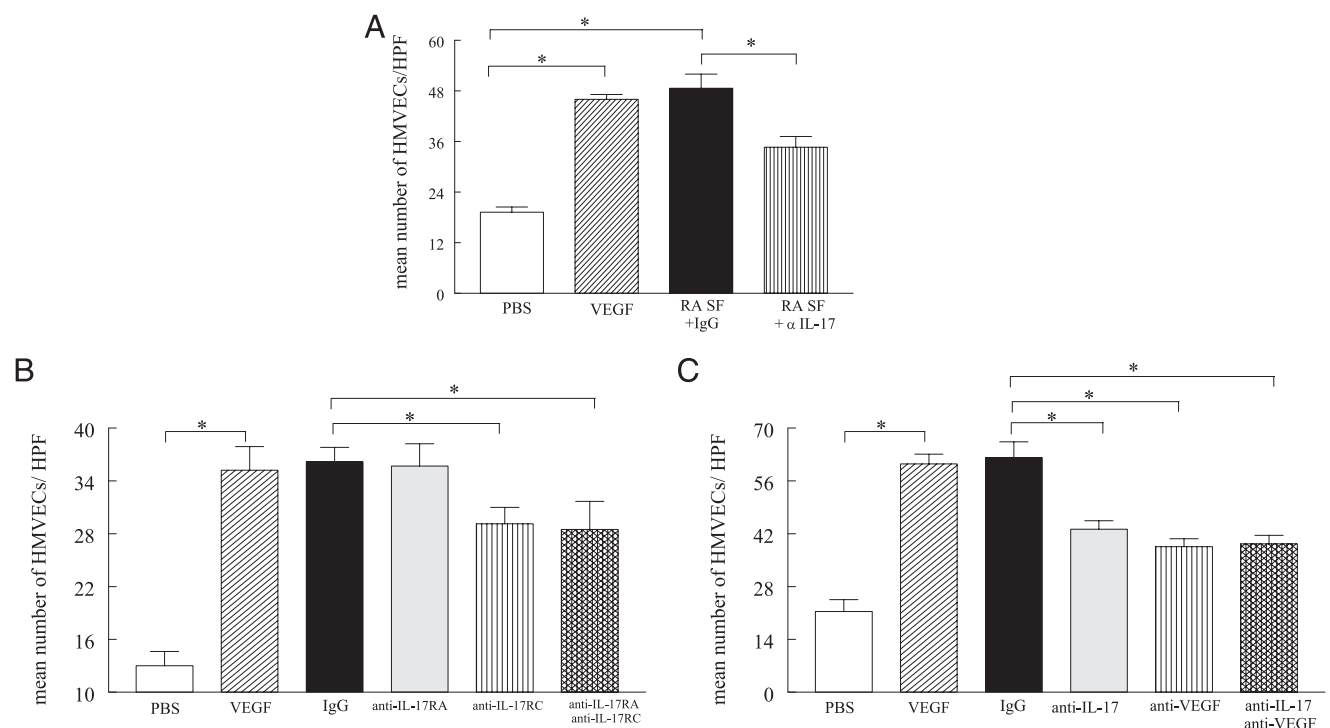
**FIGURE 6.** IL-17-induced HMVEC migration is suppressed by PI3K inhibition. To determine the mechanism of IL-17 in HMVECs, cells were stimulated with IL-17 (50 ng/ml) for 0–75 min, and the cell lysates were probed for p-AKT (*A*), pERK (*B*), or pJNK (*C*). These results are representative of three experiments. *D*, To determine signaling pathways associated with IL-17 HMVEC migration, cells were treated with the identified chemical inhibitors for PI3K (LY294002; 1 and 5  $\mu$ M) or ERK (PD98059; 1 and 5  $\mu$ M) as well as JNK (SP600125; 1, 5  $\mu$ M) 2 h in the Boyden chamber. Only inhibition of PI3K downregulated IL-17-induced HMVEC migration. Values demonstrate mean  $\pm$  SE of three experiments in triplicate. \* $p < 0.05$ .



**FIGURE 7.** IL-17-mediated tube formation is reduced by inhibition of PI3K. HMVECs were incubated with inhibitors to PI3K (LY294002; 1 and 5  $\mu$ M), ERK (PD98059; 1 and 5  $\mu$ M), JNK (SP600125; 1 and 5  $\mu$ M), or DMSO for 45 min at 37°C prior adding to polymerized Matrigel. IL-17 (50 ng/ml) was then added to the wells, and the plate was incubated for 16 h at 37°C (in triplicate). Photomicrographs (original magnification  $\times 100$ ) taken of representative wells treated with PBS (A), FGF (20 ng/ml) (B), IL-17 (50 ng/ml) plus DMSO (C), IL-17 (50 ng/ml) plus LY294002 (5  $\mu$ M) (D), IL-17 (50 ng/ml) plus PD98059 (5  $\mu$ M) (E), and IL-17 (50 ng/ml) plus SP600125 (5  $\mu$ M) (F) in which IL-17-induced tube formation is significantly reduced by the inhibition of PI3K/AKT1 pathway ( $p < 0.05$ ). G demonstrates mean number of tubes per well where LY294002 (1 and 5  $\mu$ M; PI3K/AKT1 inhibitor) significantly reduces the number of branch points induced by IL-17 activation in Matrigel tube formation assay, whereas ERK (PD98059; 1 and 5  $\mu$ M) and JNK inhibitors (SP600125; 1 and 5  $\mu$ M) were ineffective. Values are the mean  $\pm$  SE,  $n = 3$ . \* $p < 0.05$ .

PGE<sub>2</sub>, and VEGF from RA synovial fibroblasts, and the production of some of these factors is further enhanced by TNF- $\alpha$  (50). Therefore, we investigated the contribution of IL-17 to human RA synovial fluid-mediated HMVEC chemotaxis. Neutralization of IL-17 in RA synovial fluid partially reduced RA synovial fluid-mediated HMVEC chemotaxis. RA synovial fluid-mediated

HMVEC chemotaxis was mediated through IL-17RC, confirming the importance of this receptor in IL-17-mediated angiogenesis. Interestingly, angiogenic factors present in human RA synovial fluid are mostly produced by RA synovial tissue fibroblasts (VEGF, bFGF, VCAM1, IL-6, and ELR<sup>+</sup> CXC chemokines) or macrophages (TNF- $\alpha$ , IL-8, and IL-1 $\beta$ ) (25). IL-17 is the only



**FIGURE 8.** RA synovial fluid-induced HMVEC chemotaxis is mediated by IL-17 through ligation to IL-17RC, and IL-17 does not synergize with VEGF in RA synovial fluid-induced HMVEC migration. A, Anti-IL-17 (10  $\mu$ g/ml) or control IgG was added to RA synovial fluids from eight patients (1/20 dilution) (1 h at 37°C) prior to performing HMVEC chemotaxis in response to human RA synovial fluids. B, HMVECs were incubated with Abs to IL-17 RA and RC (10  $\mu$ g/ml), as well as isotype control for 1 h prior to performing HMVEC chemotaxis in response to eight human RA synovial fluids. C, RA synovial fluids from eight patients (1/20 dilution) were incubated with Abs to IL-17 (10  $\mu$ g/ml), VEGF (10  $\mu$ g/ml), or both as well as isotype control or PBS or VEGF for 1 h prior to performing HMVEC chemotaxis in response to RA synovial fluids. The values represent the mean  $\pm$  SE. \* $p < 0.05$ .

lymphokine that contributes to human RA synovial fluid-mediated angiogenesis, suggesting that T cells may also be important in this process. The data presented in this study, together with our previously reported evidence demonstrating that IL-17 is important for monocyte migration (21) in RA synovial fluids, highlight the importance of IL-17 in RA pathogenesis.

Interestingly, our results show that neutralization of IL-17 and VEGF do not synergize in reducing RA synovial fluid-induced HMVEC migration beyond the effect detected with one factor alone. As shown with IL-17, VEGF-induced HMVEC chemotaxis is mediated through PI3K (46, 51). Therefore, the lack of synergy between IL-17 and VEGF in inducing HMVEC chemotaxis may be due to that both mediators are using the same signaling pathway or that as shown previously HMVEC cell migration is within a bell shaped curve (37, 52); therefore, the synergistic effect could not be detected.

In conclusion, endothelial migration and tube formation induced by IL-17 were mediated through activation of the PI3K pathway and ligation to both IL-17 receptors. However, neutralization of only IL-17 or IL-17RC significantly downregulated human RA synovial fluid-mediated endothelial migration, suggesting that IL-17 plays an important role in RA angiogenesis.

## Disclosures

The authors have no financial conflicts of interest.

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## Local Expression of Interleukin-27 Ameliorates Collagen-Induced Arthritis

Sarah R. Pickens,<sup>1</sup> Nathan D. Chamberlain,<sup>1</sup> Michael V. Volin,<sup>2</sup> Arthur M. Mandelin II,<sup>1</sup> Hemant Agrawal,<sup>1</sup> Masanori Matsui,<sup>3</sup> Takayuki Yoshimoto,<sup>4</sup> and Shiva Shahrara<sup>1</sup>

**Objective.** To determine the mechanism of action of interleukin-27 (IL-27) against rheumatoid arthritis (RA).

**Methods.** Adenovirus containing IL-27 transcript was constructed and was locally delivered into the ankles of mice with collagen-induced arthritis (CIA). Progression of arthritis was determined in treated and untreated mice by measuring ankle circumference and through histologic analysis. IL-17 and its downstream targets as well as cytokines promoting Th17 cell differentiation were quantified by enzyme-linked immunosorbent assay in CIA mouse ankles locally expressing adenoviral IL-27 as well as in control-treated mouse ankles. Ankles from both treatment groups were immunostained for neutrophil and monocyte migration (macrophages in the tissue). Finally, vascularization was quantified by histology and by determining ankle hemoglobin levels.

**Results.** Ectopic expression of IL-27 in CIA mice ameliorated inflammation, lining hypertrophy, and bone erosion as compared with control-treated CIA mice. Serum and joint levels of IL-17 were significantly

reduced in the IL-27-treated group compared with the control-treated group. Two of the main cytokines that induce Th17 cell differentiation and IL-17 downstream target molecules were greatly down-regulated in CIA mouse ankles receiving forced expression of IL-27. The control mice had higher levels of vascularization and monocyte trafficking than did mice ectopically expressing IL-27.

**Conclusion.** Our results suggest that increased levels of IL-27 relieve arthritis in CIA mouse ankles. This amelioration of arthritis involves a reduction in CIA mouse serum and joint levels of IL-17 and results in decreased IL-17-mediated monocyte recruitment and angiogenesis. Hence, the use of IL-27 may be a strategy for treatment of patients with RA.

Interleukin-17 (IL-17) is found in rheumatoid arthritis (RA) synovial fluid and in the T cell-rich areas of RA synovial tissue (1,2). Th17 cells, which are derived from RA synovial tissue, are significantly increased in RA synovial fluid compared with RA or normal peripheral blood (3). Our recent studies have shown that IL-17 mediates angiogenesis in RA synovial fluid through ligation to IL-17 receptor C (4). IL-17 can also contribute to the pathogenesis of RA by inducing monocyte migration into the inflamed synovial tissue (5,6).

IL-17 plays a profound role in the pathogenesis of experimental arthritis. Collagen-induced arthritis (CIA) is markedly reduced in IL-17<sup>-/-</sup> mice (7), and treatment of the experimental CIA model with anti-IL-17 antibody decreases the severity of inflammation and bone destruction (8). Further, local expression of IL-17 increases inflammation and synovial lining thickness (9), which we have shown to be associated with increased vascularity and monocyte recruitment (3,6).

IL-27 is a heterodimeric cytokine produced by macrophages and dendritic cells; it belongs to the IL-12 cytokine family, which includes IL-23 and IL-35 (10). IL-27 is composed of 2 subunits, Epstein-Barr virus-

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<sup>1</sup>Sarah R. Pickens, MA (current address: University of Illinois at Chicago), Nathan D. Chamberlain, BBmE (current address: University of Illinois at Chicago), Arthur M. Mandelin II, MD, PhD, Hemant Agrawal, PhD, Shiva Shahrara, PhD (current address: University of Illinois at Chicago): Northwestern University Feinberg School of Medicine, Chicago, Illinois; <sup>2</sup>Michael V. Volin, PhD: Midwestern University, Chicago College of Osteopathic Medicine, Downers Grove, Illinois; <sup>3</sup>Masanori Matsui, PhD: Saitama Medical University, Saitama, Japan; <sup>4</sup>Takayuki Yoshimoto, PhD: Tokyo Medical University, Tokyo, Japan.

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Address correspondence to Shiva Shahrara, PhD, University of Illinois at Chicago, Department of Medicine, Division of Rheumatology, E807 MSB, 835S Wolcott Avenue, Chicago, IL 60612. E-mail: shahrara@uic.edu.

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induced gene 3 and p28, whose transcriptions are regulated independently. As such, dissociation of the expression of the 2 subunits may occur (11). Dendritic cells produce IL-27 when stimulated by pathogen-associated molecular patterns through Toll-like receptors (TLRs) (12). We have shown that macrophages from RA synovial fluid have significantly higher levels of IL-27 production compared with control cells; however, both groups of cells produced similar levels of IL-27 in the presence of TLR-2 ligation (3). Consistent with this, others have shown that IL-27 is expressed in RA synovium (13).

IL-27 mediates its proinflammatory effect by modulating the initial step of Th1 cell differentiation through the induction of IL-12 receptor  $\beta 2$  (IL-12R $\beta 2$ ) expression, which can lead to interferon- $\gamma$  (IFN $\gamma$ ) production (14,15). Concordant with these results, IL-27R $^{-/-}$  mice demonstrated reduced inflammation in the proteoglycan-induced arthritis model by down-regulating IFN $\gamma$ -producing T cells (16). In contrast, IL-27 can also suppress inflammation by inhibiting murine Th17 cell differentiation mediated by IL-6 and transforming growth factor  $\beta$  (TGF $\beta$ ). It was shown that the absence of IL-27 increased the severity of experimental autoimmune encephalomyelitis (EAE) by promoting T cell proliferation and Th17 cell differentiation (17,18). Further, EAE in IL-27R $\alpha^{-/-}$  mice was ameliorated by using antibody against IL-17 (17). The suppressive effect of IL-27 was distinct from that of IFN $\gamma$ , since EAE induced in double knockouts of IFN $\gamma$  and IL-27R $\alpha$  was more severe than in each single knockout alone. It was further shown that IL-27 is a potent suppressor of Th17 cell development in a STAT-1-dependent and IFN $\gamma$ -independent way (17,18). Others have shown that the antiinflammatory properties of IL-27 may also be due to induction of IL-10 by CD4 $^{+}$  cells through a STAT-1- and STAT-3-dependent pathway (19).

Experiments were performed to examine the mechanism by which IL-27 affects the pathogenesis of CIA. Our results demonstrated that 2 of the cytokines promoting Th17 cell differentiation, as well as downstream targets of IL-17 in macrophages and fibroblasts, were significantly reduced in ankles adenovirally expressing IL-27 compared with ankles injected with control phosphate buffered saline (PBS). Ectopic expression of IL-27 in the ankles down-regulated CIA vascularization and monocyte migration into synovial tissue compared with the control group. Using RA memory T cells, we demonstrated that while IL-27 treatment significantly reduced the percentage of Th17 cells, it had no effect on Th1 cells. These results suggest that inhibition

of Th17 cell polarization through IL-27 may be a useful RA treatment.

## MATERIALS AND METHODS

**Construction of AdIL-27.** Mouse IL-27 complementary DNA (cDNA) was obtained from p3xFLAG-IL-27 plasmid described previously by Matsui et al (20), and AdIL-27 was constructed by Welgen. Briefly, IL-27 cDNA was cloned into pCR-TOPO vector (Invitrogen) by polymerase chain reaction. Thereafter, IL-27 cDNA was released with *Bgl* II and ligated to pENT-CMV predigested with the same enzyme, and the positive clones were screened by digestion with *Bam* HI and sequenced. The pENT-IL-27 cDNA was treated with LR Clonase II enzyme (Invitrogen) and ligated to a pAdREP plasmid that contains the remaining adenovirus genome. The recombination products were transformed into *Escherichia coli* cells, and after overnight incubation, the positive clones were selected, and cosmid DNA was purified. The purified cosmid DNA (2  $\mu$ g) was digested with *Pac* I and then transfected into 293 cells with Lipofectamine 2000 according to the instructions of the manufacturer (Invitrogen). The 293 cells were grown in Dulbecco's modified Eagle's medium. The adenovirus plaques were seen 7 days after transfection. The concentration of the AdIL-27 was  $3 \times 10^{10}$  plaque-forming units (PFU) as determined by plaque assay. The control adenovirus (AdControl) used in this study was an empty pENT-CMV shuttle vector with no insert (adenovirus purchased from Welgen).

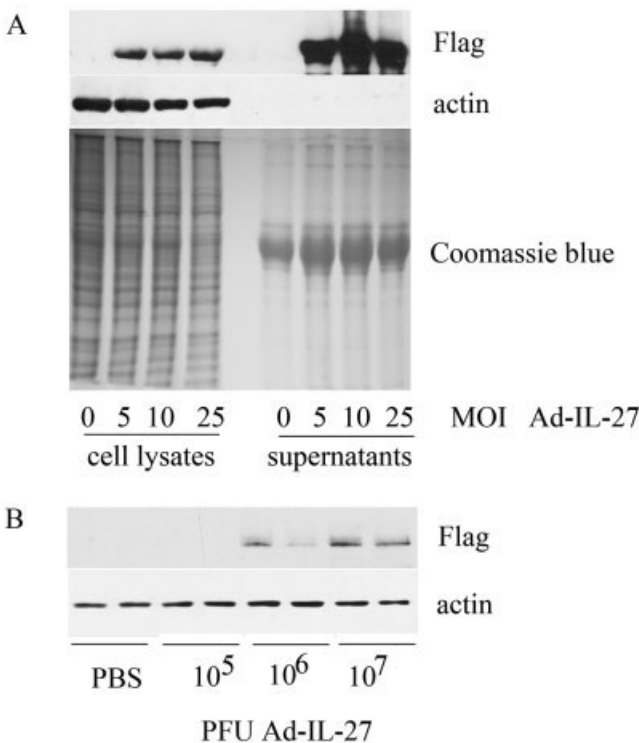
**Transfection of AdIL-27 in 293 cells and detection of AdIL-27 in mouse ankles.** We cultured 293 cells in a 6-well plate to 50–75% confluence. The next day, cells were infected at 0, 5, 10, or 25 multiplicities of infection (MOI) of AdIL-27. Following a 48-hour incubation, conditioned medium and cells were collected. The conditioned medium was concentrated using 30-kD columns (VWR Scientific Products); AdIL-27 was detected in the conditioned medium and cell lysates by probing for FLAG (1:3,000 dilution), and equal loading was determined by actin (1:3,000 dilution) or by staining with Coomassie blue. Mice were injected intraarticularly (IA) with  $10^5$ ,  $10^6$ , or  $10^7$  PFU of AdIL-27 (injected into both ankles) or with control PBS, and ankles were harvested after 5 days. Ankles were then homogenized in a 50-ml conical centrifuge tube containing 1 ml of Complete Mini-protease inhibitor cocktail (Roche Molecular Biochemicals) homogenization buffer. Ankle homogenization was completed on ice using a motorized homogenizer, followed by 30 seconds of sonication. Homogenates were centrifuged at 2,000g for 10 minutes and filtered through a 0.45- $\mu$ m pore size Millipore filter (21–23). AdIL-27 expression was examined in ankle homogenates through Western blot probing of FLAG (1:3,000 dilution), and equal loading was examined by actin (1:3,000 dilution).

**Study protocol for CIA and AdIL-27 treatment.** DBA/1J mice (age 7–8 weeks) were immunized with collagen on days 0 and 21. Bovine type II collagen (2 mg/ml; Chondrex) was emulsified in an equal volume of Freund's complete adjuvant (2 mg/ml of *Mycobacterium tuberculosis* H37Ra; Difco or Chondrex). The DBA/1J mice were immunized subcutaneously in the tail with 100  $\mu$ l of emulsion. On day 21, mice were injected intradermally with 100  $\mu$ l of type II collagen (2 mg/ml) emulsified in an equal volume of Freund's incomplete adjuvant. AdIL-27 ( $10^7$  PFU,  $n = 15$ ) or AdControl ( $10^7$  PFU,  $n = 15$ ) was injected IA on day 23 after CIA induction. Mice were

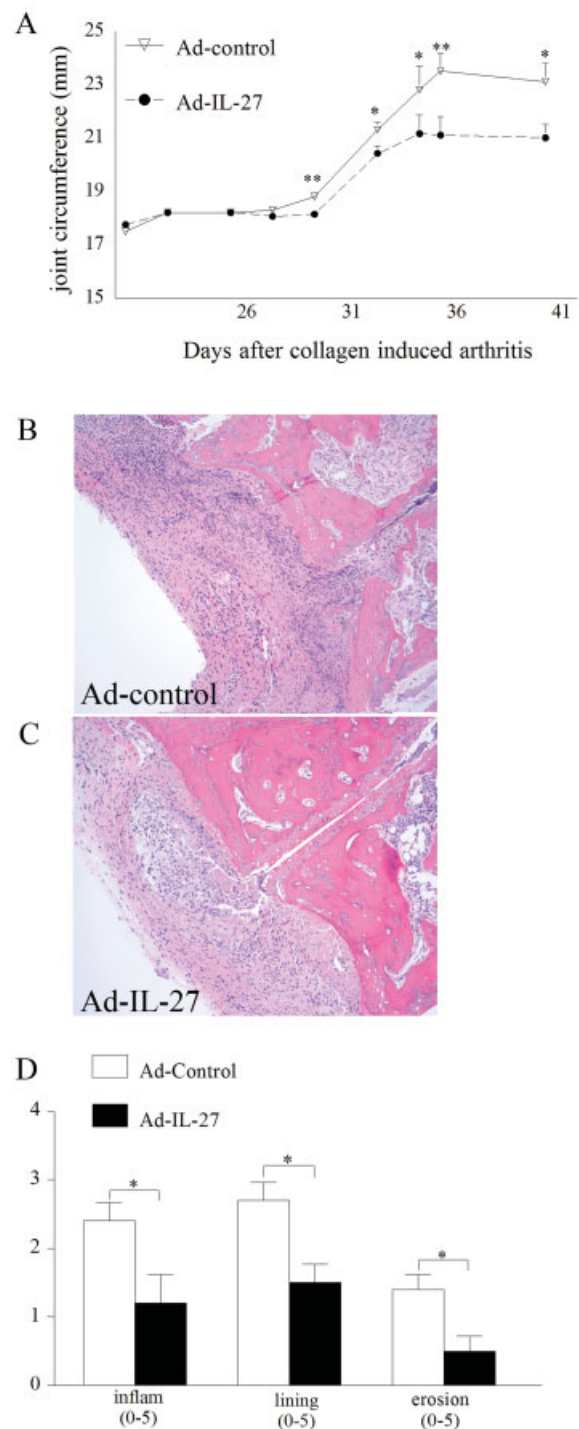
killed on day 42; ankles were harvested for protein and messenger RNA extraction, as well as for histologic studies, and serum was saved for laboratory tests.

**Clinical assessments.** Ankle circumferences were determined by measurement of 2 perpendicular diameters, including the laterolateral diameter and the anteroposterior diameter, using calipers (Lange Caliper; Cambridge Scientific). Circumference was determined using the following formula:  $\text{circumference} = 2\pi\sqrt{(a^2 + b^2)/2}$ , where *a* and *b* represent the diameters. Ankle circumference evaluations were performed on days 21, 23, 26, 28, 30, 33, 35, 36, and 41.

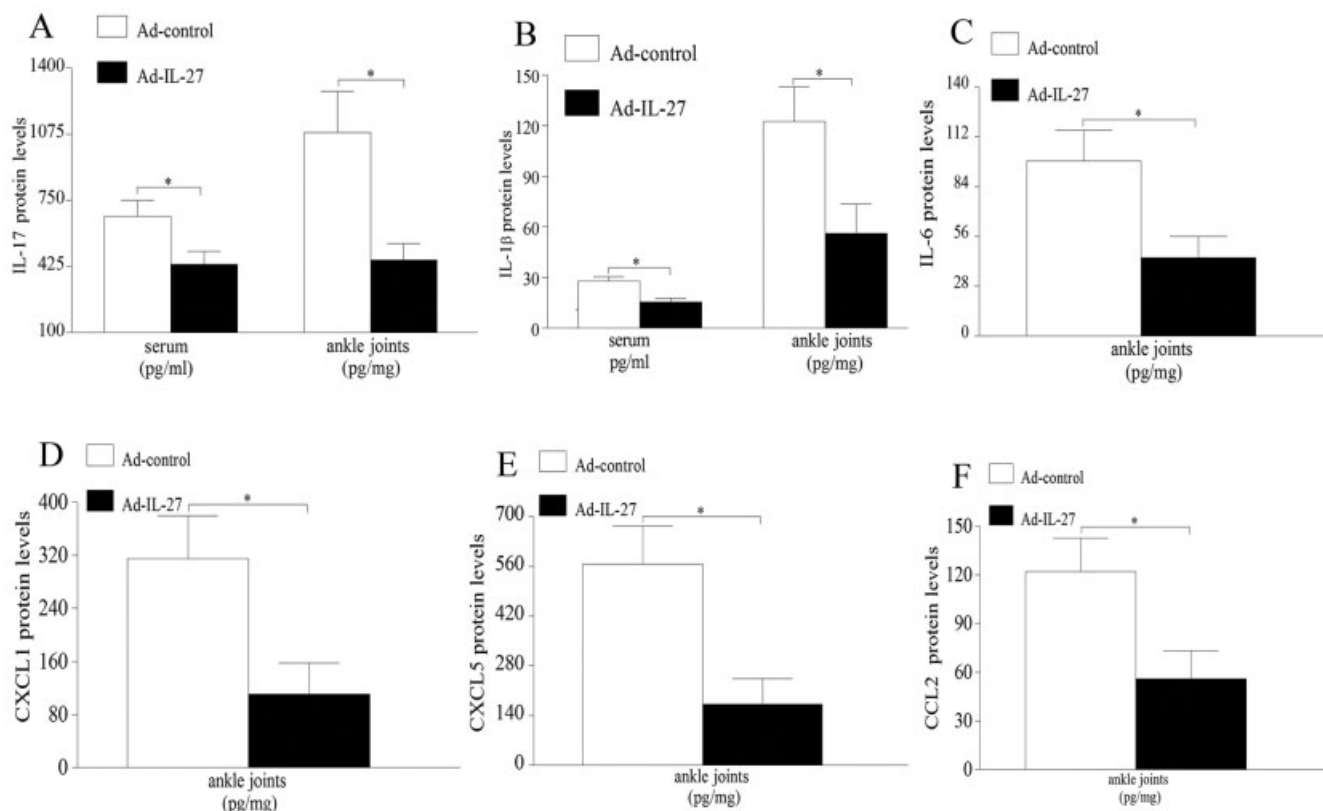
**Flow cytometry.** RA peripheral blood mononuclear cells were isolated by Histopaque gradient centrifugation, and memory CD4<sup>+</sup> T cells were isolated with a negative selection kit (StemCell Technologies) according to the manufacturer's instructions. RA memory CD4<sup>+</sup> T cells were cultured and treated with phorbol myristate acetate (PMA; 50 ng/ml) and ionomycin (1  $\mu$ g/ml), with or without IL-27 treatment (100 ng/ml) for 48 hours. The cells were supplemented with brefeldin A (10  $\mu$ g/ml) 18 hours prior to performing flow cytometry. Cells were then stained with anti-CD4 (RPA-T4; BD PharMingen), anti-IL-17 (eBio64DEC17; eBioscience), anti-IFN $\gamma$  (4S.B3; BD PharMingen), or isotype control antibodies. Th17



**Figure 1.** Western blot analysis of AdIL-27 construct. **A**, Western blot analysis using anti-FLAG, antiactin, or Coomassie blue protein staining of lysates and conditioned medium obtained from 293 cells transfected with 0, 5, 10, or 25 multiplicities of infection (MOI) of AdIL-27. **B**, Western blot analysis using anti-FLAG or antiactin antibody on homogenates of mouse ankles injected with phosphate buffered saline (PBS) control or with 10<sup>5</sup>, 10<sup>6</sup>, or 10<sup>7</sup> plaque-forming units (PFU) of AdIL-27.



**Figure 2.** Local expression of interleukin-27 (IL-27) ameliorates the pathology of collagen-induced arthritis (CIA). **A**, Changes in joint circumference. \* = *P* < 0.05; \*\* = *P* < 0.01 versus AdIL-27-treated mice. **B** and **C**, Hematoxylin and eosin staining of CIA mouse ankles injected with control adenovirus (AdControl) (**B**) or AdIL-27 (**C**). Original magnification  $\times$  200. **D**, Effect of local expression of IL-27 on inflammation, lining thickness, and bone erosion. \* = *P* < 0.05. Values in **A** and **D** are the mean  $\pm$  SEM (*n* = 10 mice).



**Figure 3.** Forced expression of IL-27 reduces levels of IL-17 and proinflammatory factors in the joint. **A** and **B**, Levels of expression of IL-17 (**A**) or IL-1 $\beta$  (**B**) in sera and ankle homogenates from CIA mice treated with AdControl or AdIL-27 were determined by enzyme-linked immunosorbent assay (ELISA). **C–F**, Levels of expression of IL-6 (**C**), CXCL1 (**D**), CXCL5 (**E**), or CCL2 (**F**) in ankle homogenates from both treatment groups were quantified by ELISA. Values are the mean  $\pm$  SEM ( $n = 9$ – $10$  mice). \* =  $P < 0.05$ . See Figure 2 for other definitions.

or Th1 cells were identified as CD4+IL-17+ or CD4+IFN $\gamma$ +, respectively.

**Antibodies and immunohistochemistry.** Mouse ankles were decalcified, fixed in formalin, embedded in paraffin, and were sectioned in the pathology core facility of Northwestern University. Inflammation, synovial lining thickness, and bone erosion (scored on a 0–5 scale) were determined by a blinded observer (AMM) viewing hematoxylin and eosin–stained sections. Mouse ankles were stained with immunoperoxidase using Vector Elite ABC Kits (Vector), with diaminobenzidine (Vector) as a chromogen, at the pathology core facility of Northwestern University.

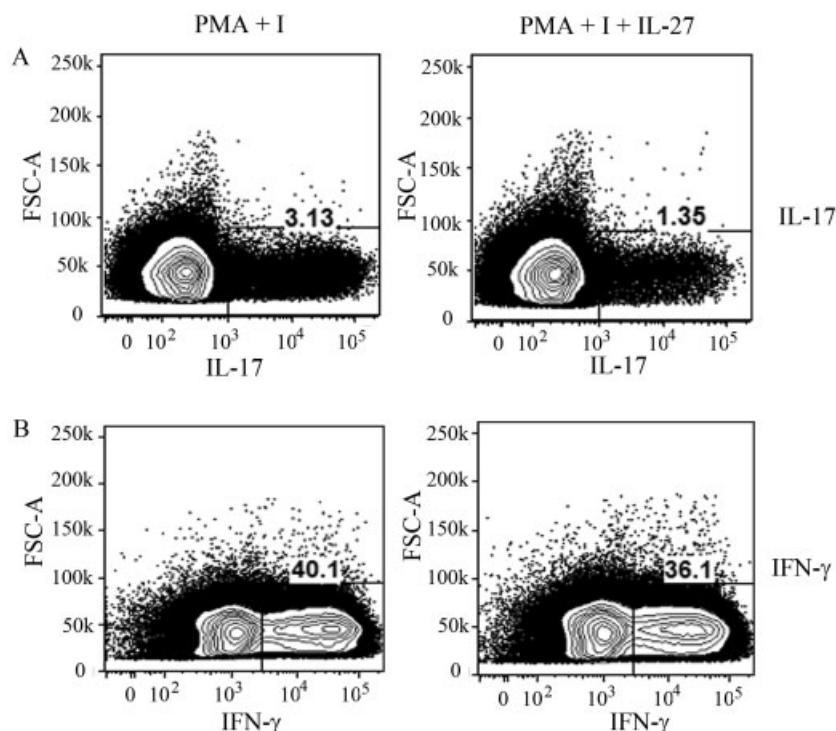
Briefly, slides were deparaffinized in xylene for 15 minutes at room temperature, followed by rehydration by transfer through graded alcohols. Antigens were unmasked by incubating slides in proteinase K digestion buffer (Dako) for 5 minutes at room temperature. Endogenous peroxidase activity was blocked by incubation with 3% H<sub>2</sub>O<sub>2</sub> for 5 minutes. Nonspecific binding of avidin and biotin was blocked using an avidin/biotin blocking kit (Dako). Nonspecific binding of antibodies to the tissues was blocked by pretreatment of tissues with Protein block (Dako). Tissues were incubated with GR1 (1:200 dilution; Novus Biologicals), MAC 387 (1:200 dilution; Serotec) together with animal research kit [ARK; Dako], von Willebrand factor (1:1,000 dilution; Dako), or control IgG

antibody (Beckman Coulter). Slides were counterstained with Mayer's hematoxylin and treated with lithium carbonate for bluing. Neutrophil and macrophage staining were scored on a 0–5 scale. Vascularity was quantified as number of blood vessels per 5 random high-power fields at 10 $\times$  magnification (24). The data were pooled, and the mean  $\pm$  SEM was calculated in each data group. Each slide was evaluated by a blinded observer (AMM) (22,23,25,26).

**Quantification of proinflammatory factors.** Mouse ankle and/or serum IL-17, IL-1 $\beta$ , IL-6, tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), CXCL1, CXCL5, CCL20, and CCL2 were quantified by enzyme-linked immunosorbent assay (ELISA) according to the instructions of the manufacturer (R&D Systems). The sensitivity of the ELISAs performed to quantify mouse IL-17, IL-1 $\beta$ , IL-6, CXCL1, and CXCL5 was 7.8 pg/ml, whereas for TNF $\alpha$  and CCL20 it was 15.6 pg/ml, and for CCL2 it was 1.95 pg/ml. The expression level of each factor was normalized to the ankle protein concentration and reported as pg/mg, and serum levels are reported as pg/ml.

**Quantification of hemoglobin in mouse ankles.** Using methemoglobin, serial dilutions were prepared to generate a standard curve from 70 gm/dl to 1.1 gm/dl (4,27,28). Fifty microliters of homogenized mouse ankles or standard was added to a 96-well plate in duplicate, and 50  $\mu$ l of tetramethylbenzidine was added to each sample. The plate was





**Figure 4.** Interleukin-27 (IL-27) treatment reduces the percentage of Th17 cells but not of Th1 cells in rheumatoid arthritis (RA) peripheral blood. **A** and **B**, Percentages of CD4+IL-17+ cells (**A**) or CD4+ interferon- $\gamma$  (IFN $\gamma$ )-positive cells (**B**) were quantified in RA peripheral blood memory CD4+ T cells treated with phorbol myristate acetate (PMA; 50 ng/ml) and ionomycin (I; 1  $\mu$ g/ml) or with PMA, ionomycin, and IL-27 (100 ng/ml) for 48 hours. The cells were supplemented with brefeldin A (10  $\mu$ g/ml) 18 hours before flow cytometry was performed. Results demonstrate that IL-27 treatment reduces Th17 cells without affecting Th1 cells. FSC = forward scatter.

allowed to develop at room temperature for 15–20 minutes with gentle shaking, and the reaction was terminated with 150  $\mu$ l of 2N H<sub>2</sub>SO<sub>4</sub> for 3–5 minutes. Absorbance was read with an ELISA plate reader at 450 nm. To calculate hemoglobin concentrations in the mouse ankles, the values (gm/dl) were normalized to the weights of the ankles (mg/ml) (4,27,28).

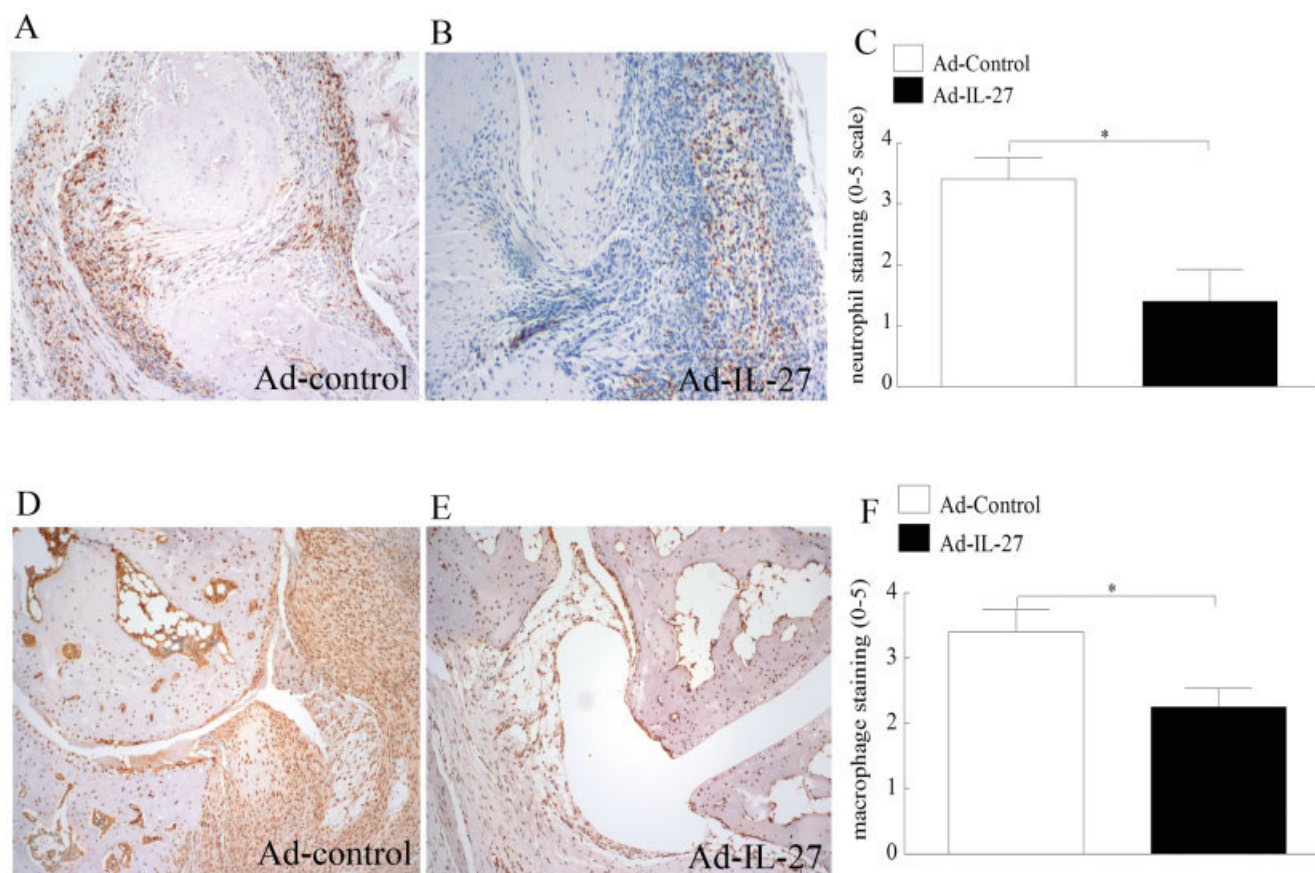
**Statistical analysis.** The data were analyzed using Student's 2-tailed *t*-tests for paired and unpaired samples. *P* values less than 0.05 were considered significant.

## RESULTS

**Expression of AdIL-27 in transfected 293 cells and in mouse ankles.** To verify that AdIL-27 was capable of expressing IL-27, 293 cells were transfected with 0, 5, 10, and 25 MOI of AdIL-27. Following a 48-hour incubation, protein expression was determined by Western blotting of both cell lysates and conditioned medium. Using anti-FLAG antibody we were able to detect AdIL-27 construct at 5, 10, and 25 MOI from both 293 cell lysates and conditioned medium (Figure 1A).

To validate the expression of IL-27 *in vivo*, mouse ankles were bilaterally injected with 10<sup>5</sup>, 10<sup>6</sup>, or 10<sup>7</sup> PFU of AdIL-27 or PBS control. After 5 days, ankles were homogenized and AdIL-27 construct was detected employing anti-FLAG antibody in Western blotting analysis. AdIL-27 construct was detectable only in ankles injected IA with 10<sup>6</sup> or 10<sup>7</sup> PFU AdIL-27, and not in ankles injected IA with 10<sup>5</sup> PFU AdIL-27 or PBS control (Figure 1B). Since both mouse ankles injected with 10<sup>7</sup> PFU AdIL-27 strongly expressed IL-27 compared with only 1 mouse ankle injected with 10<sup>6</sup> PFU AdIL-27, the dose of 10<sup>7</sup> PFU was selected for performing these experiments.

**Effect of local IL-27 expression in CIA.** To determine the effect of IL-27 administration into the arthritic joint, AdIL-27 or AdControl (10<sup>7</sup> PFU each) was injected IA into DBA/1J mouse ankles 23 days after CIA induction. In the AdControl-injected mice, disease activity determined by ankle circumference began on

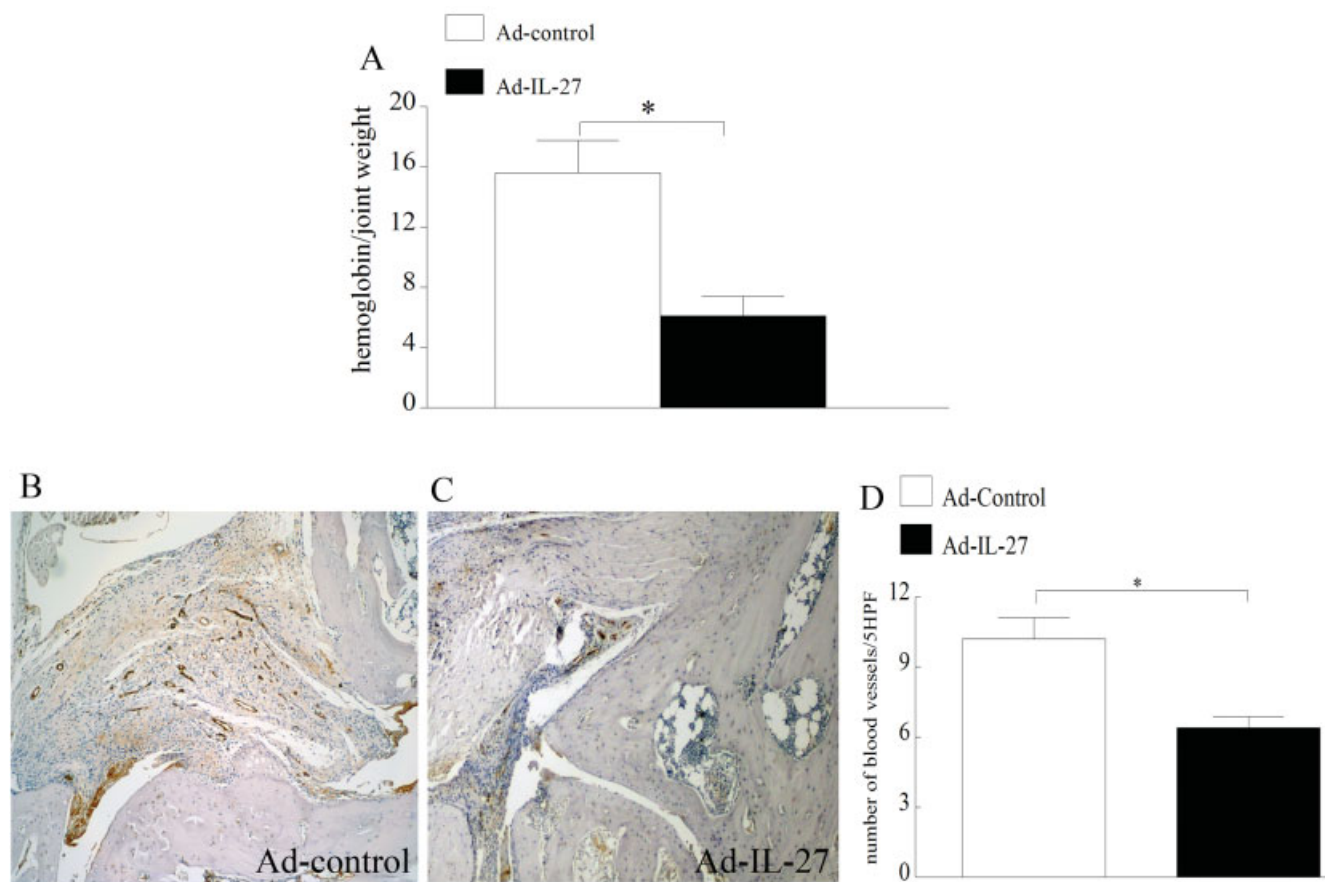


**Figure 5.** Local expression of IL-27 down-regulates CIA-mediated neutrophil and monocyte ingress. Synovial tissue from AdControl- or AdIL-27-injected CIA mouse ankles harvested on day 42 was immunostained with GR1 (neutrophil marker) (A and B) or MAC 387 (macrophage marker) (D and E). Original magnification  $\times 200$ . Neutrophil staining (C) and macrophage staining (F) of CIA mouse ankles harvested on day 42 were assessed and quantified. Values are the mean  $\pm$  SEM ( $n = 10$  mice). \* =  $P < 0.05$ . See Figure 2 for definitions.

day 30 and progressed through day 36, plateauing thereafter until the termination of the experiments on day 42 (Figure 2A). Mice treated with AdIL-27 demonstrated significantly reduced joint circumference compared with control animals ( $P < 0.05$  and  $P < 0.01$ ). Next, histologic examination of the joints was performed to determine the effect of treatment on inflammation and joint destruction. Histologic analysis of ankles obtained on day 42 confirmed that mice treated with AdIL-27 had significantly less inflammation (50% decrease), synovial lining thickness (45% decrease), and bone erosion (65% decrease) than did control mice (Figures 2B–D). These results suggest that local expression of IL-27 can reduce CIA joint inflammation, synovial lining thickness, and bone destruction.

**Effect of AdIL-27 treatment on expression of proinflammatory factors in CIA mouse ankles.** IL-27 is known to suppress inflammation by inhibiting Th17 cell differentiation; therefore, IL-17 expression levels were determined in serum and ankles of CIA mice receiving

AdIL-27 or AdControl. Our results demonstrate that IL-17 expression was significantly lowered in sera and ankle homogenates (by 35% and 55%, respectively) from AdIL-27-treated animals compared with those from control animals (Figure 3A). Interestingly, we demonstrated that 2 of the cytokines that drive Th17 cell differentiation, namely, IL-1 $\beta$  and IL-6, were significantly reduced (by 55%) in mouse ankles, and levels of IL-1 $\beta$  were also decreased (by 45%) in sera from CIA mice locally expressing IL-27 compared with sera from mice receiving control treatment (Figures 3B and C). We have shown that CXCL1, CXCL5, and CCL2 are neutrophil and monocyte chemokines that are induced by IL-17 in RA synovial tissue fibroblasts and macrophages as well as in a model of IL-17-induced arthritis (ref. 6 and Pickens SR, Chamberlain ND, Shahrara S: unpublished observations). We found that ectopic expression of IL-27 significantly decreased joint levels of CXCL1 (by 65%), CXCL5 (by 70%), and CCL2 (by 55%) compared with AdControl treatment in CIA (Fig-



**Figure 6.** Reduced vascularization detected in CIA mouse ankles locally expressing IL-27. **A**, Hemoglobin levels in CIA mouse ankles harvested from different treatment groups on day 42 were quantified. Results are shown as hemoglobin (gm/dl)/joint weight (mg/ml). **B** and **C**, Synovial tissue from AdControl-injected (**B**) or AdIL-27-injected (**C**) CIA mouse ankles harvested on day 42 was immunostained with von Willebrand factor (endothelial marker). Original magnification  $\times 200$ . **D**, Vascularization was quantified as the number of blood vessels per 5 random high-power fields (hpf) at  $10\times$  magnification in each CIA mouse ankle harvested on day 42. Values in **A** and **D** are the mean  $\pm$  SEM ( $n = 10$  mice). \* =  $P < 0.05$ . See Figure 2 for other definitions.

ures 3D–F). These results suggest that local expression of IL-27 could suppress Th17 cell polarization as well as IL-17 downstream target genes.

**Treatment with IL-27 significantly reduces RA Th17 cells without affecting Th1 cells.** Since splenocytes and T cells are difficult to transfect due to low expression of adenovirus receptor, in order to demonstrate that IL-27 can directly reduce CD4+IL-17+ cells, RA peripheral blood memory T cells were isolated by negative selection and were treated with PMA and ionomycin with or without IL-27. Results from these experiments demonstrate that while IL-27 treatment significantly reduced Th17 cells from 3% to 1%, it had no effect on the Th1 cell population (Figures 4A and B). Consistently, when IL-17 levels in conditioned medium were quantified by ELISA (after 48 and 72 hours), cells treated with IL-27 had lower secretion of IL-17 compared with cells in the control treatment group (data not shown).

**Effect of AdIL-27 treatment on leukocyte recruitment into CIA mouse joints.** We have shown thus far that local expression of IL-27 in CIA mouse ankles significantly reduced inflammation as well as Th17 cell polarizing cytokines and IL-17-induced downstream factors. We next examined the effect of AdIL-27 on leukocyte recruitment into inflamed CIA mouse ankle joints. Consistent with the clinical data, local expression of IL-27 greatly suppressed the ingress of neutrophil (by 60%) (Figures 5A–C) and monocytes (by 35%) (Figures 5D–F) into CIA mouse joints as compared with control mouse joints. Our results suggest that reduction of joint IL-17 levels can down-regulate trafficking of neutrophils and monocytes into CIA mouse ankles.

**Effect of AdIL-27 treatment on vascularization in CIA.** Since angiogenesis is critical for leukocyte ingress, the effect of local IL-27 expression on blood vessel formation in CIA mice was studied. Vascularization in CIA mice was quantified by measuring ankle hemoglo-



bin levels and blood vessel staining. AdIL-27-treated CIA mice had markedly lower hemoglobin levels compared with control mice (Figure 6A). Consistently, there were 40% fewer blood vessels in CIA mouse ankles that locally expressed IL-27 than in control mouse ankles (Figures 6B–D). Our results suggest that IL-27 treatment inhibits IL-17-mediated angiogenesis in CIA.

## DISCUSSION

In this study, we showed that local expression of IL-27 in CIA mouse ankles ameliorates joint inflammation and bone destruction. We further demonstrated that IL-27 modulates arthritis through reducing 2 important Th17 cell polarizing cytokines as well as IL-17-activated factors in the CIA mouse joint. In RA peripheral blood, IL-27 treatment directly reduced the percentage of Th17 cells; however, Th1 cells were unaffected. Consequently, local expression of IL-27 in CIA mouse ankles suppresses IL-17-mediated neutrophil and monocyte trafficking as well as vascularization. These results suggest that IL-27 can inhibit IL-17-induced acute (neutrophil migration) and chronic (monocyte recruitment) inflammation by affecting leukocyte ingress, controlled in part by the reduction in angiogenesis.

Early neutralization of IL-17 using an IL-17 receptor IgG Fc fusion protein in CIA suppresses the onset of the disease (29). Treatment of CIA after disease onset using anti-IL-17 antibody decreases the severity of inflammation and bone destruction in CIA (8). These studies demonstrated that IL-17 plays an important role in the initiation and progression of CIA. Hence, we investigated whether inhibition of Th17 cell differentiation could reduce joint inflammation in CIA. The effect of local expression of IL-27 on Th17 cells was observed both systemically and in the ankle joints, since IL-17 levels in the sera and ankle homogenates were markedly decreased compared with those in the control group. Experiments were performed in RA peripheral blood in order to demonstrate that IL-27 treatment could directly inhibit Th17 cell differentiation and that reduction in IL-17 levels was distinct from IFN $\gamma$ -mediated Th17 cell suppression. Consistent with previous findings (17,18), we showed that Th17 cell differentiation was suppressed 3-fold while Th1 cell polarization was unaffected by IL-27 treatment in RA peripheral blood.

We found that local expression of IL-27 could alleviate clinical signs of CIA. Consistent with this, histologic analysis demonstrated reduced inflammation, synovial lining thickness, and bone erosion, which may be due to suppressed joint levels of IL-17. It has been

shown that IL-17 is involved in bone degradation through elevating the expression of RANKL in CIA mouse ankles (30) as well as through synergizing with TNF $\alpha$  and IL-6 in this process (31,32).

TGF $\beta$ , IL-6, IL-1 $\beta$ , and IL-21 drive the differentiation of Th17 cells (33–35). However, some variation between humans and mice has been described. Levels of IL-1 $\beta$  and IL-6, but not TNF $\alpha$ , were markedly reduced in CIA mouse ankles locally expressing IL-27 compared with control mouse ankles. Others have shown that in IL-1Ra<sup>-/-</sup> mice, elevated levels of IL-1 $\beta$  are responsible for an increase in the number of Th17 cells (36). In CIA, IL-6 is essential for Th17 cell differentiation, since anti-IL-6R antibody markedly suppresses induction of Th17 cells and arthritis development (37). Consistent with our data, a previous study demonstrated that systemic administration of recombinant IL-27 could reduce serum levels of IL-6 (13). Interestingly, in CIA mouse synoviocytes, neutralization of IL-1 $\beta$  and IL-6 significantly reduces IL-17-mediated expression of TLRs 2, 4, and 9 (38). The results from our laboratory and others suggest that IL-1 $\beta$  and IL-6 are 2 of the cytokines that play an important role in Th17 cell differentiation in CIA. Therefore, IL-27 can suppress polarization of Th17 cells by modulating joint levels of IL-1 $\beta$  and IL-6.

Our unpublished studies demonstrated that IL-17 can induce CXCL1 expression from RA synovial tissue fibroblasts, macrophages, and human microvascular endothelial cells. CXCL5 is also produced from IL-17-activated RA synovial tissue fibroblasts and macrophages (Pickens SR, Chamberlain ND, Shahrara S: unpublished observations). Previous studies have shown that neutrophil migration mediated by IA injection of IL-17 is dependent on CXCL1 and CXCL5, suggesting that both neutrophil chemokines are produced by cells in the ankle joints and play an essential role in IL-17-mediated neutrophil ingress (39). Neutrophil chemotaxis caused by conditioned media from IL-17-stimulated gastric epithelial cells was inhibited by neutralizing antibodies to IL-8, suggesting that in human cells IL-8 is responsible for IL-17-induced neutrophil trafficking (40). Similar to IL-8, CXCL1 and CXCL5 bind to CXCR2 and therefore may induce neutrophil migration through activation of the same pathway. Collectively, the data suggest that reduction of IL-17 by AdIL-27 suppresses neutrophil migration through modulating CXCL1 and CXCL5 in CIA mouse ankle joints.

We have shown that IL-17 plays an important role in monocyte migration in RA, since neutralization of IL-17 in RA synovial fluid or its receptors on monocytes significantly reduces monocyte migration mediated by RA synovial fluid (5). Further, IL-17 promotes mono-

cyte migration through activation of p38 MAPK (5). We also found that IL-17 activates CCL2 production by macrophages and RA synovial tissue fibroblasts and in experimental arthritis models (6). In addition to the direct effect of IL-17 on monocyte chemotaxis, we demonstrated that IL-17-mediated monocyte recruitment into the peritoneal cavity was due in part to CCL2 production (6). Despite the ability of IL-17 to induce the production of other monocyte chemokines, such as CCL20, from cells present in the synovial lining (6), forced expression of IL-27 in CIA mouse ankles did not affect the expression levels of this chemokine. Based on our previous studies, inhibition of monocyte recruitment into CIA mouse ankles locally expressing IL-27 may be directly due to reduction of IL-17 levels or indirectly due to lower expression of IL-17-induced CCL2, or perhaps both mechanisms are essential for this process.

Angiogenesis is an early and critical event in the pathogenesis of RA, which is triggered by the inflammatory process mediated by cytokines, chemokines, and hypoxia (41). Previous studies demonstrated that angiogenesis is essential for CIA progression (42). In the current study, we showed that local expression of IL-27 significantly reduced synovial vascularity in CIA mice compared with that in control animals. This effect may be due to down-regulation of joint levels of IL-17 in ankles with forced IL-27 expression. We previously observed that IL-17, in concentrations present in the RA joint, induces endothelial migration through the phosphatidylinositol 3-kinase/Akt-1 pathway (4). Further, we have demonstrated that IL-17 is angiogenic, as determined by its ability to promote blood vessel growth in Matrigel plugs *in vivo* (4). However, reduced levels of the proangiogenic chemokines CXCL1 and CXCL5 may also be responsible for the decreased vascularity in the AdIL-27 treatment group compared with that in the control group (43). Given that angiogenesis promotes the ingress of leukocytes, reduction in new blood vessel formation can affect neutrophil and monocyte trafficking.

In summary, local expression of IL-27 in CIA results in reduced disease severity quantified by joint swelling, synovial lining thickness, bone erosion, and leukocyte migration. In CIA, AdIL-27 treatment leads to reduced IL-1 $\beta$  and IL-6 production, resulting in a depressed Th17 cell response characterized by decreased joint levels of IL-17. This leads to decreased synovial production of the neutrophil and monocyte chemokines CXCL1, CXCL5, and CCL2, ultimately resulting in fewer infiltrating leukocytes and less blood vessel formation.

## AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Shahrara had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Pickens, Shahrara.

**Acquisition of data.** Pickens, Chamberlain, Volin, Mandelin, Agrawal, Shahrara.

**Analysis and interpretation of data.** Pickens, Chamberlain, Volin, Matsui, Yoshimoto, Shahrara.

**Providing reagents.** Matsui, Yoshimoto.

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## Anti-CXCL5 therapy ameliorates IL-17-induced arthritis by decreasing joint vascularization

Sarah R. Pickens · Nathan D. Chamberlain · Michael V. Volin ·  
Mark Gonzalez · Richard M. Pope · Arthur M. Mandelin II ·  
Jay K. Kolls · Shiva Shahrara

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**Abstract** IL-17-induced joint inflammation is associated with increased angiogenesis. However, the mechanism by which IL-17 mediates angiogenesis is undefined. Therefore, the pathologic role of CXCL1 and CXCL5 was investigated in arthritis mediated by local expression of IL-17, employing a neutralizing antibody to each chemokine. Next, endothelial chemotaxis was utilized to examine whether endothelial migration was differentially mediated by CXCL1 and CXCL5. Our results demonstrate that IL-17-mediated disease activity was not affected by anti-CXCL1 treatment alone. In contrast, mice receiving anti-CXCL5 demonstrated significantly reduced clinical signs of arthritis, compared to the mice treated with IgG control. Consistently, while inflammation, synovial lining thickness, bone erosion and vascularization were markedly

reduced in both the anti-CXCL5 and combination anti-CXCL1 and 5 treatment groups, mice receiving anti-CXCL1 antibody had clinical scores similar to the control group. In contrast to joint FGF2 and VEGF levels, TNF- $\alpha$  was significantly reduced in mice receiving anti-CXCL5 or combination of anti-CXCL1 and 5 therapies compared to the control group. We found that, like IL-17, CXCL1-induced endothelial migration is mediated through activation of PI3K. In contrast, activation of NF- $\kappa$ B pathway was essential for endothelial chemotaxis induced by CXCL5. Although CXCL1 and CXCL5 can differentially mediate endothelial trafficking, blockade of CXCR2 can inhibit endothelial chemotaxis mediated by either of these chemokines. These results suggest that blockade of CXCL5 can modulate IL-17-induced inflammation in part by reducing joint blood vessel formation through a non-overlapping IL-17 mechanism.

S. R. Pickens · N. D. Chamberlain · S. Shahrara (✉)  
Division of Rheumatology, Department of Medicine, University  
of Illinois at Chicago, MSB 835S Wolcott Ave., E807-E809,  
Chicago, IL 60612, USA  
e-mail: shahrara@uic.edu

M. V. Volin  
Department of Microbiology and Immunology, Chicago College  
of Osteopathic Medicine, Midwestern University, Downers  
Grove, IL 60515, USA

M. Gonzalez  
Orthopaedic Surgery, University of Illinois at Chicago,  
Chicago, IL 60612, USA

R. M. Pope · A. M. Mandelin II  
Department of Medicine, Feinberg School of Medicine,  
Northwestern University, Chicago, IL 60611, USA

J. K. Kolls  
Department of Genetics, LSU Health Sciences Center,  
New Orleans, LA 70112, USA

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### Introduction

RA is an autoimmune disease in which angiogenesis can promote ingress of leukocytes, as well as pannus formation, thereby perpetuating inflammation and bone destruction [1]. Although RA was initially considered to be a TH-1-mediated disease, recent studies from experimental arthritis models indicate that TH-17 cells play a crucial role in the initiation and progression of the disease [2–5]. As such, the incidence and severity of collagen-induced arthritis (CIA) were significantly alleviated in IL-17-deficient mice, and post-onset blockade of IL-17 ameliorates CIA inflammation and joint destruction [6]. Further, local expression of



IL-17 exacerbates disease in the CIA [4] and K/BxN serum transfer arthritis models [3]. Not only can IL-17 amplify disease severity in experimental arthritis models, but its local expression can also mediate joint inflammation and synovial lining thickness in naive mice [3].

It has been shown that the proinflammatory activity of IL-17 is imparted by its ability to induce neutrophil ingress and granulopoiesis [7–9]. In studies using human neutrophils, migration induced by IL-17 was inhibited by a neutralizing antibody to IL-8, suggesting that IL-17-induced neutrophil migration is mediated through IL-8 production [10].

Previous studies have shown that IL-17-activated RA synovial tissue fibroblasts produce a number of CXC chemokines [11, 12] that are known to be neutrophil chemoattractant and proangiogenic. Further CXCL1 and CXCL5 mRNA transcripts are modulated by IL-17 through enhanced stabilization [12–14]. Chemokines such as CXCL1, 2, 3, 5 and 6, are corresponding ligands to CXCR2, and are important proangiogenic factors in RA joints [15–17] that can activate Matrigel tube formation and angiogenesis [18, 19]. Although blockade of CXCR1/CXCR2 in experimental arthritis models ameliorates joint inflammation by inhibiting adhesion and migration of neutrophils, the efficacy and the mechanism of the corresponding ligands are undefined [20–22].

Our recent studies demonstrate that IL-17 contributes to angiogenesis in RA since neutralization of IL-17 in RA synovial fluid or IL-17 receptor C (RC) on human microvascular endothelial cells (HMVECs) significantly reduces RA synovial fluid induced endothelial migration [23]. We also show that vascularity was increased in an IL-17-induced arthritis model [23].

In the current study, we examined the hypothesis that IL-17-mediated arthritis may be due to elevated chemokine levels that promote angiogenesis. To find these important factors, we screened IL-17-activated macrophages, RA synovial tissue fibroblasts and HMVECs for proangiogenic chemokine expression. Elevated proangiogenic chemokine expression was validated in the IL-17-induced arthritis model. Although expression of several factors was identified in these cell types/tissues, CXCL1 and CXCL5 were the most highly expressed in IL-17-activated RA synovial tissue explants and the experimental arthritis model. To demonstrate the pathologic role of CXCL1 and CXCL5 in IL-17-mediated arthritis, neutralizing antibodies to each chemokine were employed. We found that arthritis severity and vascularization were significantly reduced in the anti-CXCL5 treatment group. In contrast, anti-CXCL1 treatment had no effect on IL-17-mediated disease activity or neovascularization, while being capable of inhibiting CXCL1-mediated endothelial chemotaxis in vitro. The combination of anti-CXCL1 and anti-CXCL5 was not more

effective than anti-CXCL5 treatment alone. We next demonstrated that ligation to CXCR2 facilitates CXCL1 and CXCL5-induced endothelial migration although downstream signaling pathways are differentially regulated by these chemokines. We show that while CXCL1 can induce endothelial migration through activation of PI3K pathway, this process was mediated by CXCL5 through NF- $\kappa$ B signaling. Since both CXCL1 and IL-17 can mediate angiogenesis through the same pathway, blocking of CXCL1 is ineffective in this process whereas suppression of CXCL5 can effectively reduce NF- $\kappa$ B mediated angiogenesis. In conclusion, these observations suggest that IL-17-mediated joint vascularization may be in part due to CXCL5 induction.

## Materials and methods

### Cell and tissue treatment for mRNA studies

The studies were approved by the Institutional Ethics Review Board and all donors gave informed written consent. Since the RA synovial tissues were recruited from the practices of orthopedic surgeons these samples are de-identified and therefore the disease severity and the treatment information is unavailable. RA synovial tissue fibroblasts were isolated from fresh RA synovial tissues, who fulfilled the American College of Rheumatology criteria for RA [24], by mincing and digesting in a solution of dispase, collagenase, and DNase [25–27]. Cells were used between passages 3–9. RA synovial tissue fibroblasts were treated with IL-17 (50 ng/ml) from 0 to 8 h for mRNA studies. Also, RA synovial tissue fibroblasts were either untreated or treated with IL-17 (50 ng/ml), TNF- $\alpha$  (10 ng/ml) or IL-17 plus TNF- $\alpha$  for 8 h. Monocytes were separated from buffy coats (Lifesource, Chicago, IL) obtained from healthy donors [26, 28]. Mononuclear cells, isolated by Histopaque (Sigma-Aldrich, St. Louis, MO) gradient centrifugation, were separated by countercurrent centrifugal elutriation. Monocytes were allowed to differentiate to macrophages as previously described [26, 28]. Macrophages were treated with IL-17 (50 ng/ml) from 0 to 8 h for mRNA studies. To determine IL-17-induced proangiogenic factors in RA synovial tissue, RA synovial tissue explants were activated with IL-17 (100 ng/ml) or PBS for 24 h. Thereafter, tissues were harvested and homogenized and protein levels of CXCL1, CXCL5, FGF2 and VEGF were determined by ELISA and results were shown as fold increase above RA synovial tissue explants treated with PBS. To define which signaling pathways mediate IL-17-induced CXCL1 or CXCL5 secretion, macrophages or RA fibroblasts were either untreated or incubated with DMSO or inhibitors to PI3K (LY294002; 10  $\mu$ M), ERK (PD98059; 10  $\mu$ M), JNK

(SP600125; 10  $\mu$ M) or p38 (SB203580; 10  $\mu$ M) for 1 h in serum free RPMI. Cells treated with DMSO or inhibitors were subsequently activated with IL-17 (50 ng/ml) for 24 h and the media was collected in order to quantify the levels of CXCL1 or CXCL5 employing ELISA.

#### Real-time RT-PCR

Macrophages and RA synovial tissue fibroblasts were treated as mentioned in the figure legends and total cellular RNA was extracted using trizol (Invitrogen, Carlsbad, CA). Subsequently, reverse transcription and real-time RT-PCR was performed as previously described [23, 28, 29]. Relative gene expression was determined by the  $\Delta\Delta C_t$  method normalized to GAPDH values, and results were shown as fold increase above 0 h and/or PBS treatment.

#### Tissue homogenization

Mouse ankles were homogenized as described previously [29, 30] in 1 ml of Complete Mini protease-inhibitor cocktail homogenization buffer (Roche, Indianapolis, IN) on ice, followed by sonication for 30 s. Homogenates were centrifuged and filtered through a 0.45  $\mu$ m pore size filter before quantifying the levels of various cytokines and chemokines by ELISA.

#### Cytokine quantification

Mouse CXCL1, CXCL5, FGF2, VEGF, IL-1 $\beta$ , CCL2, CCL3, CCL5, CCL20, IL-1 $\beta$ , IL-6 and TNF- $\alpha$  (R&D Systems, Minneapolis, MN) ELISA kits were used according to the manufacturers' instructions.

#### In vivo study protocol

The animal studies were approved by the Institutional Animal Care and Use Committee. Experiments were performed to determine the joint expression levels of CXCL1 and CXCL5 in an IL-17-induced arthritis model. For this purpose, 6–7-week-old C57BL/6 mice were injected intra-articularly with  $10^7$  PFU adenoviral (Ad)-IL-17 or Ad-control [23, 27]. Ankles were harvested on days 4 and 10 post-Ad-IL-17 or Ad-control injection, and joint CXCL1, CXCL5, FGF2 (day 10) and VEGF (day 10) levels were quantified by ELISA. In a different set of studies, experiments were performed to determine whether CXCL1 and/or CXCL5 play a role in arthritis mediated by local IL-17 expression in mouse ankle joints. For this purpose, 6–7-week-old C57BL/6 mice were treated intraperitoneally with 30  $\mu$ g (total of 210  $\mu$ g was utilized in the course of treatment) of either IgG, monoclonal rat anti-mouse

CXCL1, monoclonal rat anti-mouse CXCL5 or both anti-CXCL1 and anti-CXCL5 antibodies (at the concentrations of 1–2.5  $\mu$ g/ml, anti-CXCL1 and anti-CXCL5 are capable of neutralizing 50% (ND50) of mouse CXCL1 and CXCL5 (at 30 ng/ml); Leinco Technologies, St. Louis, Missouri) on days -4, -2, 0, 3, 5, 7 and 9 post-Ad injection with each group containing 10–12 mice. On day 0, Ad-IL-17 ( $10^7$  PFU) was injected intra-articularly into the mouse ankle joints in each treatment group. Joint circumferences were measured on days 0, 3, 5, 7 and 10 post-Ad-IL-17 injection. On day 11, post-injection ankles were harvested for ELISA and immunohistochemical studies, and blood was collected by cardiac puncture to measure blood cell count using a HemaVet 850 complete blood counter (Drew Scientific, Waterbury, CT).

#### Clinical assessments

Ankle circumferences were determined by measurement of two perpendicular diameters, the latero-lateral diameter and the antero-posterior diameter, using a caliper (Lange Caliper; Cambridge Scientific Industries). Circumference was determined using the following formula: circumference =  $2\pi \times (\text{sqrt}(a^2 + b^2/2))$  [31, 32].

#### Abs and immunohistochemistry

Mouse ankles were decalcified with ethylenediamine tetraacetic acid (Sigma, St. Louis, MO) in 10% formalin for 3 weeks, formalin fixed and paraffin embedded, and sectioned in the pathology core facility. Inflammation, synovial lining and bone erosion (based on a 0–5 score) [33] were determined using H&E-stained sections by a blinded observer (A.M.M.). Mouse ankles were immunoperoxidase-stained using Vector Elite ABC Kits (Vector Laboratories, Burlingame, CA), with diaminobenzidine (Vector Laboratories) as a chromogen by the pathology core facility. Briefly, slides were deparaffinized in xylene for 15 min at room temperature, followed by rehydration by transfer through graded alcohols. Antigens were unmasked by incubating slides in Proteinase K digestion buffer (Dako, Carpinteria, CA) for 5 min at room temperature. Endogenous peroxidase activity was blocked by incubation with 3%  $\text{H}_2\text{O}_2$  for 5 min. Nonspecific binding of avidin and biotin was blocked using an avidin/biotin blocking kit (Dako, Carpinteria, CA). Nonspecific binding of antibodies to the tissues was blocked by pretreatment of tissues with Protein block (Dako). Tissues were incubated with Von willebrand factor (1:1,000 dilution; Dako) or control IgG antibody (Beckman Coulter, Brea, CA). Slides were counterstained with Mayer's hematoxylin and treated with lithium carbonate for bluing. Endothelial staining was scored on a 0–5 scale where 0 = no staining, 1 = few cells

stained, 2 = some (less than half) cells stained, 3 = around half of the cells were stained positively, 4 = majority or more than half of the cells were positively stained, and 5 = all cells were positively stained. Data were pooled, the mean  $\pm$  SEM was calculated and each slide was evaluated by a blinded observer (A.M.M.) [31, 32, 34, 35].

**Characterization of CXCL1 and CXCL5 activated signaling pathways in human microvascular endothelial cells (HMVECs)**

HMVECs (passage 3–8) (Lonza, Walkersville, Maryland) were grown to 80% confluence in EGM-2 MV bullet kit (Lonza) and were incubated in endothelial basal medium (EBM) (Lonza) with 0% FBS for 2 h prior to treatment. Cells were then untreated or treated with CXCL1 or CXCL5 (20 ng/ml) for 5–65 min. Cell lysates were examined by Western blot analysis, as previously described [25, 26, 28]. Blots were probed with I $\kappa$ B, phospho (p)-p38, pAKT and pERK (Cell Signaling; 1:1,000 dilution) overnight or probed with actin, p38, AKT or ERK (Sigma or Cell Signaling; 1:3,000 dilution) for 1 h.

**Examining the mechanism of CXCL1 and CXCL5-induced HMVEC migration**

To examine chemotaxis, HMVECs were incubated in EBM (Lonza) with 0% FBS and no growth factors for 2 h before use. HMVECs ( $2.7 \times 10^4$  cells/25  $\mu$ l EBM with 0.1% FBS) from different treatments were then placed in the bottom wells of a 48-well Boyden chemotaxis chamber (NeuroProbe, Cabin John, MD) with gelatin-coated polycarbonate membranes (8  $\mu$ m pore size; Nucleopore, Pleasant, CA) [23, 36]. To define which signaling pathway(s) mediated CXCL1 and CXCL5-induced HMVEC chemotaxis, HMVECs were incubated with DMSO or inhibitors to PI3K (LY294002; 1 and 5  $\mu$ M), ERK (PD98059; 1 and 5  $\mu$ M), p38 (SB203580; 1 and 5  $\mu$ M) or NF- $\kappa$ B (MG-132; 1 and 5  $\mu$ M) at 37°C for 2 h, allowing endothelial cell attachment to the membrane [23]. The chamber was reinverted, and PBS, positive control VEGF (10 ng/ml; R&D Systems), CXCL1 (20 ng/ml; R&D Systems) or CXCL5 (20 ng/ml; R&D Systems) was added to the upper wells, and the chamber was further incubated for 2 h at 37°C. To examine whether CXCR2 is involved in CXCL1 and CXCL5-mediated HMVEC migration, HMVECs were incubated with antibody to CXCR2 (10  $\mu$ g/ml, R&D systems; at 37°C for 2 h while cells were attaching to the membrane) and chemotaxis was examined in response to CXCL1 or CXCL5 (1 and 20 ng/ml; for 2 h at 37°C). Readings represent fold increase chemotaxis above cells migrating in response to PBS (cells were read

in three high power  $\times 40$  fields/well, averaged for each triplicate wells and subsequent values are shown as fold increase above PBS values from two different chemotaxis assays).

**Statistical analysis**

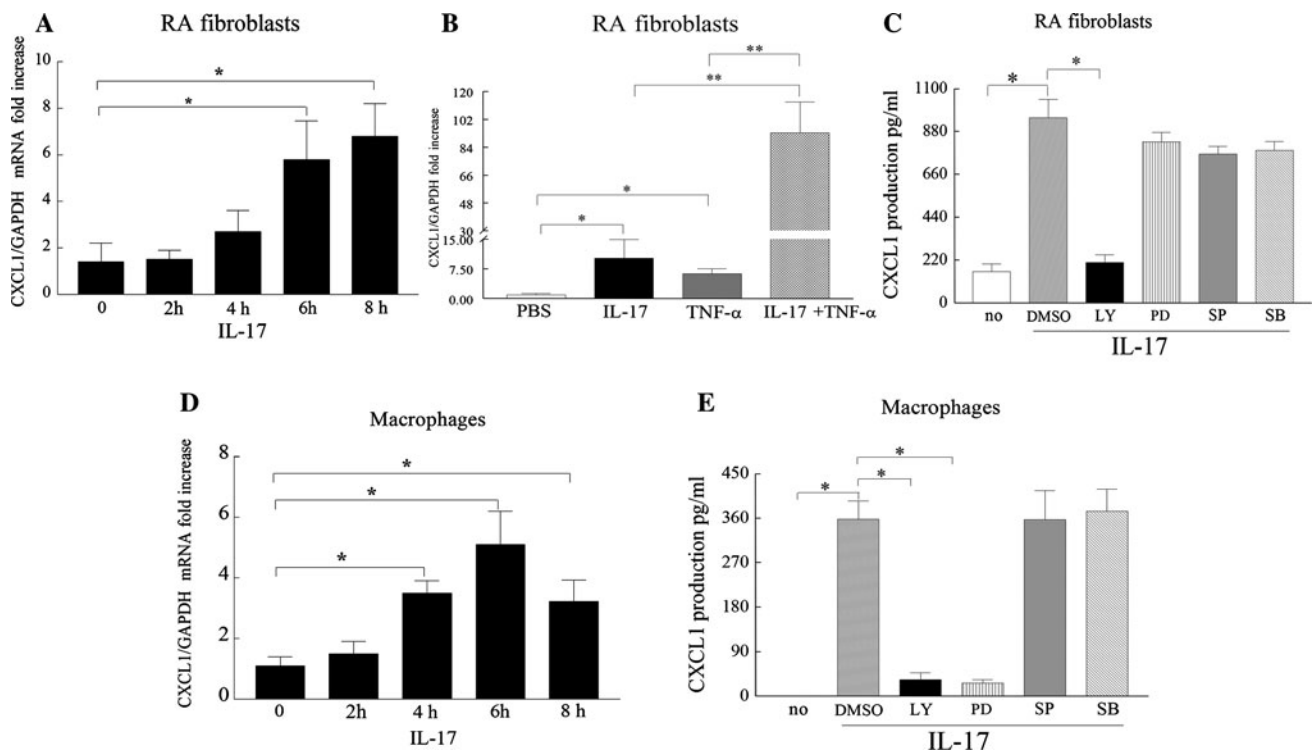
The data was analyzed employing 1-way ANOVA followed by a post hoc two-tailed Student's *t* tests for paired and unpaired samples. Values of *P* < 0.05 were considered significant.

## Results

IL-17 induces the expression of CXCL1 and CXCL5 from cells present in the RA joint through activation of PI3K and/or ERK pathway and IL-17 synergizes with TNF- $\alpha$  in inducing the expression of CXCL1 and CXCL5 in RA synovial tissue fibroblasts

IL-17-induced downstream targets were determined employing RA synovial tissue fibroblasts, macrophages differentiated in vitro from monocytes and endothelial cells, because these cells are important in the pathogenesis of RA. We found that RA synovial tissue fibroblasts and peripheral blood differentiated macrophages that are activated with IL-17 express higher levels of CXCL1 and CXCL5 (*P* < 0.05) starting at 4 h or 6 h post-stimulation (Figs. 1a, 1d, 2a, 2d), compared to control treatment. Further, only the expression of CXCL1 was significantly upregulated in HMVECs activated by IL-17 as early as 2 h post-stimulation, compared to controls (data not shown). Our previous studies demonstrate that in macrophages and RA synovial tissue fibroblasts IL-17 signals through ERK, p38 and AKT while it only activates JNK pathway in RA synovial tissue fibroblasts [27]. To determine the mechanism by which IL-17 induces CXCL1 and CXCL5 production, these pathways were suppressed in RA synovial tissue fibroblasts and macrophages activated by IL-17. Our data demonstrate that inhibition of PI3K and ERK pathways suppress production of CXCL1 in macrophages and CXCL5 in both cell types (Figs. 1e, 2c, 2e). However, in RA fibroblasts only inhibition of PI3K was capable of reducing IL-17-mediated CXCL1 levels (Fig. 1c).

Interestingly, RA synovial tissue fibroblasts activated with IL-17 and TNF- $\alpha$  demonstrate significantly greater levels of CXCL1 (Fig. 1b) and CXCL5 (Fig. 2b), compared to cells activated with IL-17 or TNF- $\alpha$  alone. However, this synergistic effect was not detected in macrophages or when RA synovial tissue fibroblasts were stimulated with IL-17 and IL-1 $\beta$  (data not shown). Our results suggest that CXCL1 and CXCL5 may be important



**Fig. 1** IL-17 induces production of CXCL1 in RA synovial fibroblasts and macrophages however, only in RA fibroblasts is CXCL1 expression synergistically induced by IL-17 and TNF- $\alpha$ . RA synovial tissue fibroblasts (**a**) and normal macrophages (**d**) were activated with IL-17 (50 ng/ml) for 0–8 h. Real-time RT-PCR was employed to identify CXCL1 (**a** and **d**) mRNA levels which were normalized to GAPDH. The results are presented as fold increase, compared with the 0 h time point (untreated cells). RA synovial tissue fibroblasts (**c**) and normal macrophages (**e**) were either untreated or incubated with DMSO or inhibitors to PI3K (LY294002; 10  $\mu$ M), ERK (PD98059; 10  $\mu$ M), JNK (SP600125; 10  $\mu$ M) or p38 (SB203580;

10  $\mu$ M) for 1 h. Thereafter cells treated with DMSO or inhibitors were subsequently activated with IL-17 (50 ng/ml) for 24 h and the media was collected from all conditions in order to quantify the levels of CXCL1 employing ELISA. **b** RA synovial tissue fibroblasts were either unstimulated or stimulated with IL-17 (50 ng/ml), TNF- $\alpha$  (10 ng/ml), or IL-17 plus TNF- $\alpha$  for 6 h. Cells were harvested, and CXCL1 mRNA levels were quantified by real-time RT-PCR which were normalized to GAPDH and presented as fold increase above PBS treatment (unstimulated cells). Values represent the mean  $\pm$  SE. \* Represents  $P < 0.05$  and \*\* denotes  $P < 0.01$ ,  $n = 3$ –5

downstream mediators expressed by RA synovial cells in response to IL-17 stimulation, and that TNF- $\alpha$  stimulation further promotes IL-17 induction of these chemokines.

CXCL1 and CXCL5 are elevated in RA synovial tissue explants and IL-17-induced arthritis model

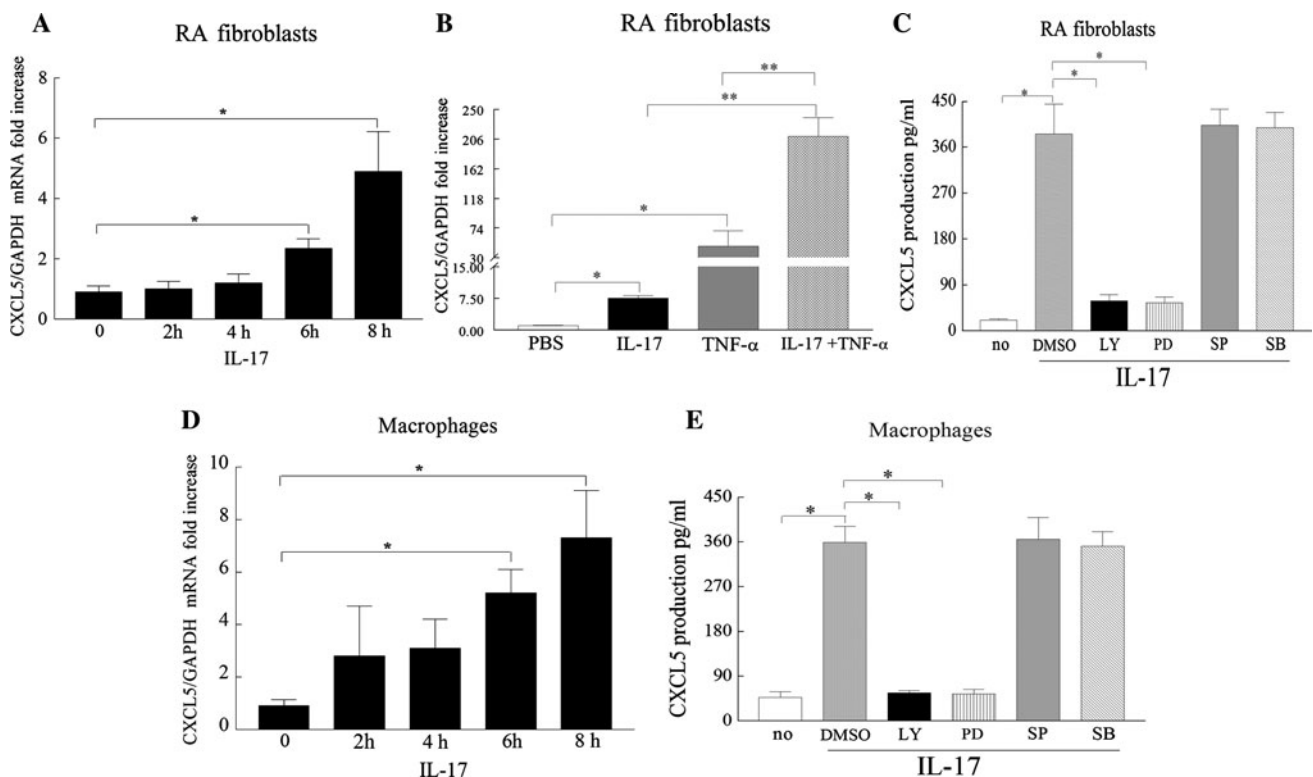
In order to determine the IL-17 modulated proangiogenic factors in RA synovial tissue explants and IL-17-induced arthritis model, levels of CXCL1, CXCL5, FGF2 and VEGF were quantified in IL-17 activated RA synovial tissue explants and/or IL-17-mediated arthritis ankles (harvested from day 10 post injection) and the data were demonstrated as fold increase above the control group (Fig. 3a, b). The results obtained from IL-17-induced arthritis model are similar to our finding in RA synovial tissue explants in that CXCL1 and CXCL5 are induced to a greater extent (40–10 fold increase in IL-17-induced arthritis ankles and 7–12 fold increase in RA explants

compared to the control group) compared to FGF2 (3–2 fold increase respectively), while VEGF was not significantly elevated in any of the mentioned models. Although in the IL-17-induced arthritis model the relative increase levels above Ad-control is greater for CXCL1 (40 fold) compared to CXCL5 (tenfold) the absolute joint concentrations for CXCL1 (1,600 pg/ml) and CXCL5 (1,520 pg/ml) are comparable in day 10 post injection (Fig. 3c, d). Based on these results we concluded that CXCL1 and CXCL5 may be important in IL-17 mediated pathogenesis in RA and this experimental arthritis model.

#### Inhibition of CXCL5 but not CXCL1 ameliorates IL-17-induced arthritis

Experiments were performed to determine whether CXCL1 and/or CXCL5 play a role in arthritis mediated by local IL-17 expression in mice ankle joints. In mice locally injected with IL-17 (and IgG control), disease activity determined





**Fig. 2** In RA synovial fibroblasts and macrophages, IL-17 induces production of CXCL5 however only in RA fibroblasts is CXCL5 expression synergistically induced by IL-17 and TNF- $\alpha$  stimulation. RA synovial tissue fibroblasts (**a**) and normal macrophages (**d**) were activated with IL-17 (50 ng/ml) for 0–8 h. Real-time RT-PCR was employed to identify CXCL5 (**a** and **d**) mRNA levels which were normalized to GAPDH. The results are presented as fold increase, compared with the 0 h time point (untreated cells). RA synovial tissue fibroblasts (**c**) and normal macrophages (**e**) were either untreated or incubated with DMSO or inhibitors to PI3K (LY294002; 10  $\mu$ M), ERK (PD98059; 10  $\mu$ M), JNK (SP600125; 10  $\mu$ M) or p38

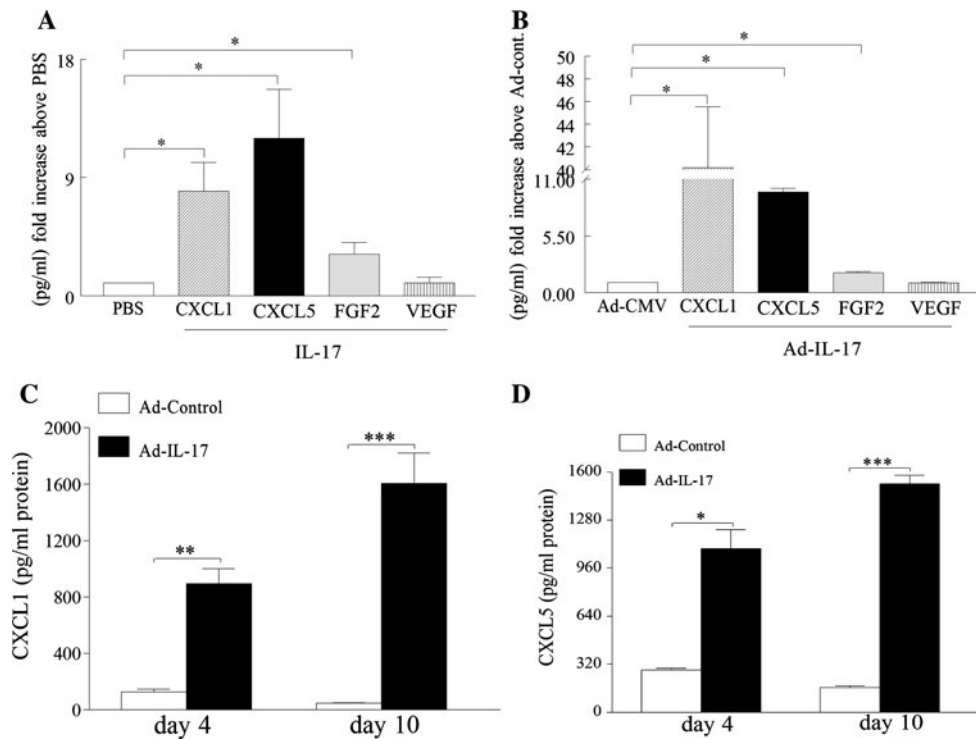
(SB203580; 10  $\mu$ M) for 1 h. Thereafter cells treated with DMSO or inhibitors were subsequently activated with IL-17 (50 ng/ml) for 24 h and the supernatants were collected from all conditions in order to quantify the levels of CXCL5 by ELISA. **b** RA synovial tissue fibroblasts were either unstimulated or stimulated with IL-17 (50 ng/ml), TNF- $\alpha$  (10 ng/ml), or IL-17 plus TNF- $\alpha$  for 6 h. Cells were harvested and CXCL5 mRNA levels were quantified by real-time RT-PCR which were normalized to GAPDH and presented as fold increase above PBS treatment (unstimulated cells). Values represent the mean  $\pm$  SE. \* Represents  $P < 0.05$  and \*\* denotes  $P < 0.01$ ,  $n = 3$ –5

by ankle circumference began around day 3 and progressed through day 5, plateauing thereafter until the termination of the experiments on day 10 (Fig. 4a). The disease activity determined by ankle circumference was significantly lower in mice receiving anti-CXCL1 on days 3 and 5, compared to the control group. However, as the arthritis progressed there was no difference noted at later time points (days 7 and 10) (Fig. 4a). In vitro chemotaxis performed on endothelial cells demonstrated that the anti-CXCL1 antibody could markedly suppress CXCL1-induced endothelial migration while anti-CXCL5 antibody did not have any effect on this process (data not shown). Further, mice receiving anti-CXCL5 demonstrated significantly reduced clinical signs of arthritis at all time points, compared to the mice treated with IgG control ( $P < 0.05$ ). The combination of anti-CXCL1 and anti-CXCL5 did not ameliorate IL-17-induced joint inflammation beyond the effect observed using anti-CXCL5 alone. Next, histological examination of

the joints was performed to determine the effect of treatment on inflammation, synovial lining and joint destruction. Consistent with the clinical data, histological analysis of the treatment groups demonstrated that inflammation, synovial lining thickening, and bone erosion were markedly reduced in the anti-CXCL5 and anti-CXCL1 and anti-CXCL5 treatment groups. In contrast, mice receiving anti-CXCL1 antibody had similar clinical scores compared to the control group (Fig. 4b, c). Our results demonstrate that CXCL5, and not CXCL1, plays an important role in IL-17-mediated arthritis.

#### Anti-CXCL5 treatment downregulates proinflammatory mediators in IL-17-induced arthritis model

To determine the role of CXCL1 and CXCL5 on IL-17-induced arthritis, proinflammatory mediators were quantified



**Fig. 3** IL-17 increases the expression of CXCL1 and CXCL5 in RA synovial tissue explants and experimental arthritis model. **a** RA synovial tissue explants were treated with PBS or IL-17 (100 ng/ml), tissues were harvested after 24 h and levels of CXCL1, CXCL5, FGF2 and VEGF were quantified by ELISA and normalized to PBS values. **b** Ad-IL-17 or Ad-CMV control ( $10^7$  PFU) was injected intra-articularly into C57/BL6 mice. Ankles were harvested on day 10 and

levels of CXCL1, CXCL5, FGF2 and VEGF were measured by ELISA and normalized to Ad-CMV. **c, d** Ankles from Ad-IL-17 and Ad-CMV treatment were harvested on days 4 and 10, and CXCL1 (**c**) and CXCL5 (**d**) levels were quantified by ELISA. Values are reported as mean  $\pm$  SE. \* Denotes  $P < 0.05$ , \*\* denotes  $P < 0.01$  and \*\*\* denotes  $P < 0.005$ ,  $n = 8-10$

in ankle joints. For this purpose, the effect of therapy was examined on joint TNF- $\alpha$ , IL-6, IL-1 $\beta$ , CCL2, CCL3, CCL5, CCL20, CXCL2, FGF2 and VEGF protein levels. Our results demonstrate that mice receiving anti-CXCL5 or combination therapy had 40–50% lower levels of joint TNF- $\alpha$ , compared to the control group (Fig. 4d). Joint CCL5 levels were also significantly (40–50%) reduced in IL-17-induced arthritis ankles receiving anti-CXCL5 or combination of anti-CXCL1 and 5, respectively (data not shown). Other joint proinflammatory mediators such as IL-6, IL-1 $\beta$ , CCL2, CCL3, CCL20, and CXCL2 were not affected by anti-CXCL5, anti-CXCL1 or combination treatments (data not shown). To demonstrate that the efficacy of anti-CXCL5 treatment is independent of the reduction of potent proangiogenic factors, joint FGF2 and VEGF were quantified in all four treatment groups. The data demonstrate that although levels of FGF2 but not VEGF are elevated in IL-17-induced arthritis model, ankles treated with anti-CXCL1 and anti-CXCL5 have similar levels of FGF2 (Fig. 5a) and VEGF (Fig. 5b) suggesting that anti-CXCL5 treatment can directly suppress IL-17-mediated angiogenesis. These results demonstrate that neutralization of CXCL5

modulates joint TNF- $\alpha$  and CCL5 levels in IL-17-mediated arthritis model.

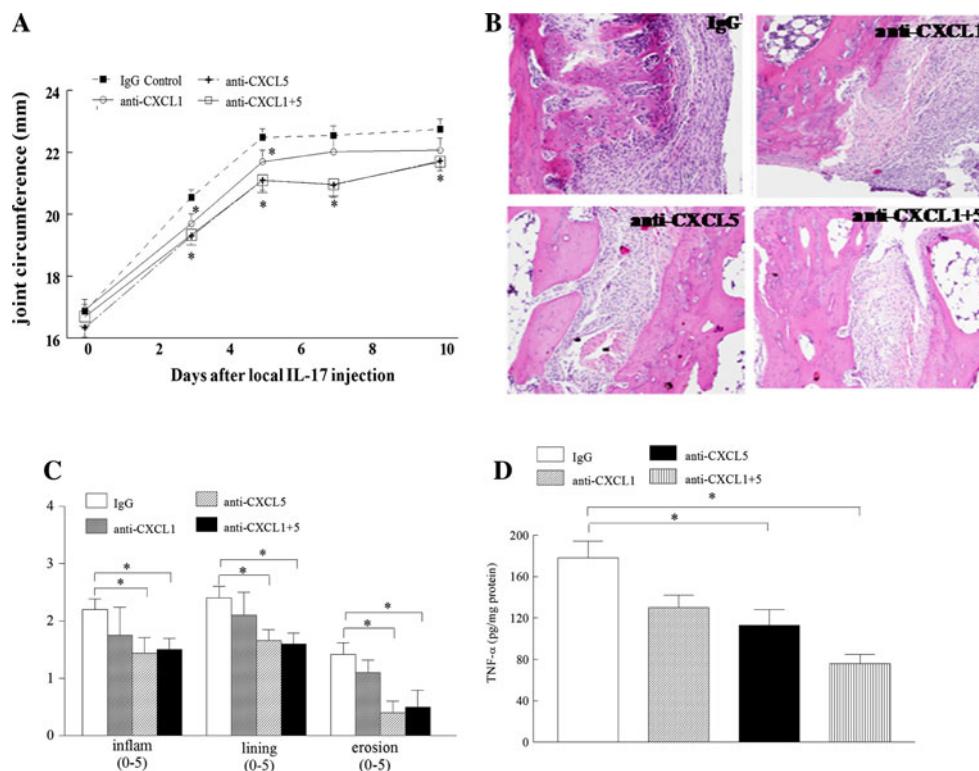
#### Anti-CXCL5 treatment reduces IL-17-induced vascularization

To determine the mechanism by which anti-CXCL5 ameliorates IL-17-induced arthritis, ankles were examined for joint vascularization. The data demonstrate that while levels of vascularization were similar in the IgG and the anti-CXCL1 treatment groups, anti-CXCL5 and the combination therapy had 40% fewer blood vessels (Fig. 5c, d). Our results may suggest that neutralization of CXCL5 can affect IL-17-induced arthritis through reduced blood vessel formation.

#### Number of blood leukocytes, neutrophils and monocytes were unaffected in anti-CXCL1 and 5 treatments

To determine whether the IL-17-induced arthritis model could be affected by systemic treatment with anti-CXCL1





**Fig. 4** Neutralization of CXCL5 ameliorates IL-17-induced joint inflammation, synovial lining thickness, bone erosion and joint TNF- $\alpha$  levels. C57BL/6 mice were treated intraperitoneally with 30  $\mu$ g (total of 210  $\mu$ g was utilized over the course of treatment) IgG, anti-CXCL1, anti-CXCL5 or both anti-CXCL1 and 5 antibodies (Leinco Technologies) on days -4, -2, 0, 3, 5, 7 and 9 post-Ad injection. On day 0, Ad-IL-17 ( $10^7$  PFU) was injected intra-articularly into the mouse ankle joint, and the joint circumference (**a**) was measured on

days 0, 3, 5, 7 and 10 post-Ad-IL-17 injection and each experimental group consisted of 10–12 mice. **b, c** Inflammation, synovial lining and bone erosion (based on a 0–5 score) were determined using H&E-stained sections by a blinded observer,  $n = 10$  ankles. Changes in the levels of joint TNF- $\alpha$  (**d**) were measured in ankle homogenates obtained from different treatment groups by ELISA and were normalized by protein concentration,  $n = 7$ –9 ankles. Values demonstrate mean  $\pm$  SE. \* Denotes  $P < 0.05$

and/or anti-CXCL5, the number of leukocytes, neutrophils and monocytes were measured in mouse whole blood. Interestingly, all treatment groups had similar numbers of white blood cells, neutrophils and monocytes (Fig. 6a), in contrast to lower levels of joint neutrophils in the anti-CXCL5 and combination therapy (data not shown). These results suggest that although the number of circulating cells was unchanged in the anti-CXCL5 and combination therapy groups, fewer cells migrated into these IL-17-mediated arthritis joints (as shown in H&E staining in Fig. 4b, c).

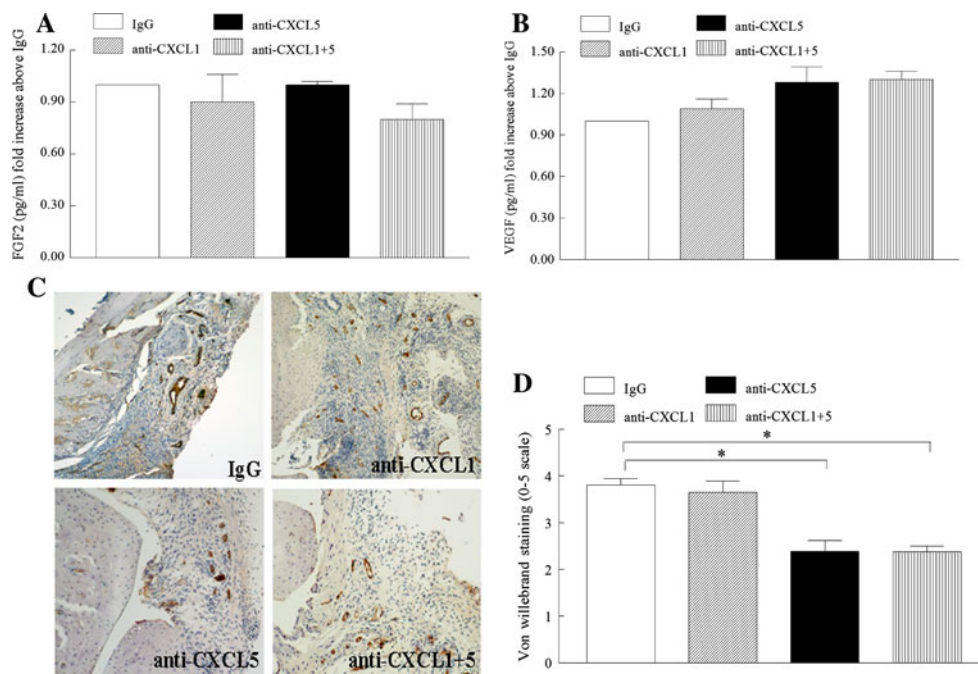
#### CXCL1 and CXCL5 mediate endothelial migration through CXCR2 ligation

To demonstrate whether CXCR2 ligation is involved in CXCL1 and CXCL5 function, CXCR2 on endothelial cells was blocked employing anti-CXCR2 neutralizing antibody and subsequently endothelial chemotaxis was examined in response to CXCL1 and CXCL5. Results from these experiments demonstrate that neutralization of CXCR2 on HMVECs significantly decreases CXCL1 (50%) and

CXCL5 (40–50%)—mediated endothelial migration (Fig. 6b) suggesting that both chemokines require CXCR2 ligation in order to mediate chemotaxis despite them signaling through different signaling pathways.

#### CXCL1 and CXCL5 induce endothelial migration through different signaling pathways

To address the different efficacy of blocking CXCL1 and CXCL5 in IL-17 experimental arthritis model we examined the mechanism by which these chemokines induce endothelial migration. We found that in HMVECs, CXCL1 signals through PI3K (35 min) and ERK (35 min) however this chemokine was unable to activate NF- $\kappa$ B or p38 signaling pathways (Figs. 7a). CXCL5 stimulation of HMVECs results in activation of NF- $\kappa$ B pathway (65 min) only (Figs. 7c). To demonstrate the mechanism by which CXCL1 and CXCL5 mediate HMVEC migration, inhibitors to these pathways were employed in in vitro chemotaxis. Interestingly while inhibition of PI3K suppresses CXCL1-induced HMVEC migration, chemotaxis mediated



**Fig. 5** Levels of joint FGF2 and VEGF were unaffected by anti-CXCL5 and combination therapy and anti-CXCL5 therapy downregulates IL-17-induced joint vascularization. Changes in the levels of joint FGF2 (a) and VEGF (b) were measured in ankle homogenates obtained from different treatment groups by ELISA and the data is presented as fold increase above the control IgG treatment,  $n = 7-9$  ankles. IL-17-induced arthritis ankles treated with IgG, anti-CXCL1,

anti-CXCL5 or the combination therapy were harvested on day 11 and were immunostained with Von willebrand factor (endothelial marker) (c) (original magnification  $\times 200$ ). Quantification of endothelial (d) staining from IL-17-induced arthritis ankles harvested on day 11,  $n = 8-9$  ankles. Values demonstrate mean  $\pm$  SE. \* Denotes  $P < 0.05$

by CXCL5 was reduced through NF- $\kappa$ B inhibition (Figs. 7b, d). These results suggest that endothelial migration is differentially regulated by CXCL1 and CXCL5.

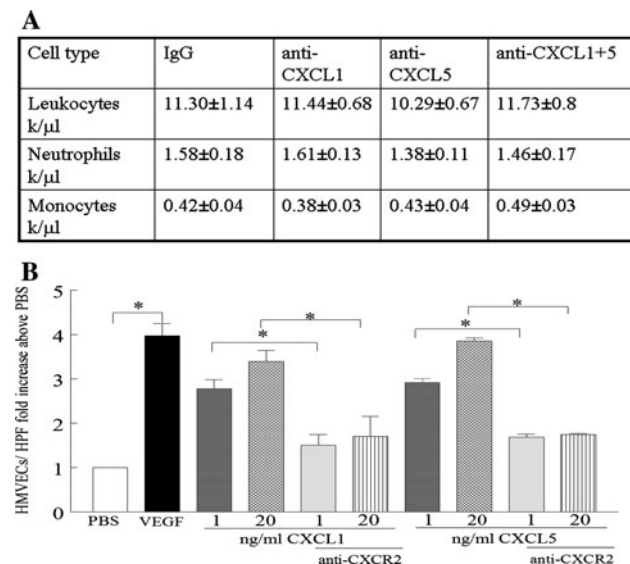
## Discussion

In this study, we show that CXCL1 and CXCL5 are important downstream mediators of IL-17 in RA synovial cells, RA synovial tissue explants and the IL-17-induced arthritis model. Neutralization of CXCL5, but not CXCL1, ameliorates joint inflammation, bone destruction and vascularization mediated by local expression of IL-17. The differential effect of CXCL1 and CXCL5 blockade in IL-17-induced arthritis model may be due to CXCL5 mediating endothelial migration through a nonoverlapping pathway with IL-17 and CXCL1 despite both chemokines ligation to CXCR2. These results suggest that differential regulation of angiogenesis by CXCL5 can suppress IL-17-induced joint inflammation.

To determine IL-17 downstream targets, RA synovial tissue fibroblasts, macrophages and HMVECs were employed. We found that genes highly induced by IL-17 were potent proangiogenic factors. We also demonstrated

that while IL-17-stimulated RA synovial tissue fibroblasts and macrophages express elevated levels of CXCL1 and CXCL5, activated HMVECs demonstrated only higher CXCL1 expression (data not shown). Consistent with our data, others have shown that CXCL1 and CXCL5 expression levels are significantly elevated in IL-17-activated preosteoblast cell line MC3T3-E1 [12]. In macrophages, CXCL1 and CXCL5 are similarly induced by IL-17 through PI3K and ERK pathways. However in RA fibroblasts, CXCL1 and CXCL5 production is differentially regulated by IL-17. Consistently, others have shown that activation of PI3K pathway plays an important role in IL-17-induced CXC chemokine expression in bronchial epithelium cells [37]. In contrast, previous studies demonstrate that IL-17-mediated CXCL1 and 2 expression in RA fibroblasts is suppressed by inhibition of p38 pathway [38]. The inconsistency in the data may be due to differences in passage number, growth condition, methods employed for quantifying mRNA levels (authors determined mRNA after 24 h treatment) [38] or patient treatment employed in the donated RA synovial tissues.

Interestingly, in RA synovial tissue fibroblasts, IL-17 and TNF- $\alpha$ , but not IL-1 $\beta$ , synergize in inducing the expression of CXCL1 and CXCL5. The amplifying effect of IL-17 and TNF- $\alpha$  in RA synovial tissue fibroblasts was



**Fig. 6** Anti-CXCL5 treatment did not affect the circulating number of leukocytes, neutrophils and monocytes in IL-17-mediated arthritis model and CXCL1 and CXCL5 mediate endothelial migration through CXCR2 ligation. **a** On day 11 blood was collected by cardiac puncture of IL-17-induced arthritis ankles treated with IgG, anti-CXCL1, anti-CXCL5 or the combination therapy to measure blood cell count using a HemaVet 850 complete blood counter. Values are shown in thousands of cells per microliter of blood (k/ $\mu$ l,  $n = 10$ –12 mice). **b** HMVECs incubated with antibody to CXCR2 (10  $\mu$ g/ml, R&D systems) were kept at 37°C for 2 h while cells were attaching to the membrane and chemotaxis was examined in response to CXCL1 and CXCL5 (1 and 20 ng/ml; for 2 h at 37°C),  $n = 2$ . Values represent fold increase chemotaxis above cells migrating in response to PBS shown as mean  $\pm$  SE of two experiments in triplicate. \* Represents  $P < 0.05$

very specific to proangiogenic chemokines, and the same effect was not detected for monocyte chemokines such as CCL2 (data not shown). Additionally, this synergistic effect on CXCL1 and CXCL5 was not noted when macrophages or HMVECs were activated with IL-17 and TNF- $\alpha$ , suggesting that the effect was specific to RA synovial tissue fibroblasts. Consistently, others have shown that in RA fibroblasts, IL-17 can synergize with TNF- $\alpha$  and IL-1 $\beta$  in inducing the production of CCL21 [39]. In nonmyeloid cells IL-17-induced stabilization of CXCL1 is independent of AUUUA motif [40] however activator of NF- $\kappa$ B1 protein (Act1) is required for this process [41]. While TNF- $\alpha$  mediated transcription of CXC chemokines is driven by NF- $\kappa$ B, this process is modulated by IL-17 through stabilizing the mRNA in an Act1 dependent manner [41]. Hence the synergy between TNF- $\alpha$  and IL-17 may reflect their independent effects on CXC chemokines.

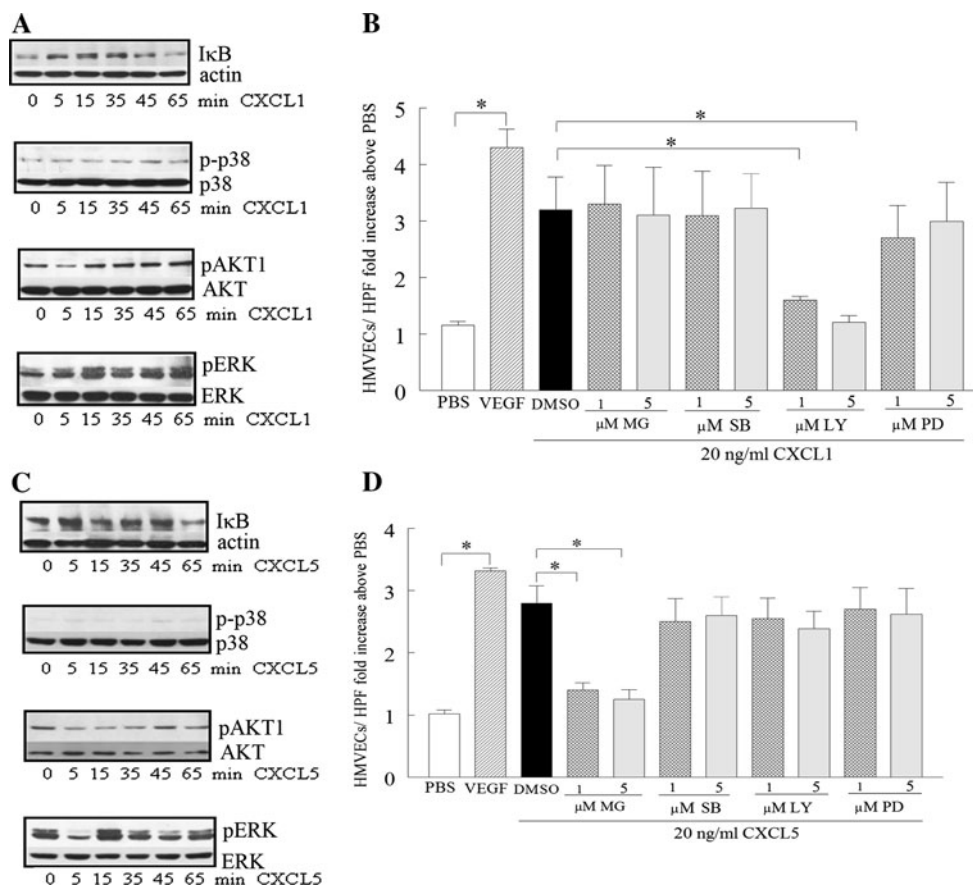
Since both CXCL1 and CXCL5 were significantly elevated in RA synovial tissue explants and IL-17-induced arthritis model to a greater extent than other proangiogenic factors such as FGF2 and VEGF, we asked whether neutralization of one or both of these chemokines could

alleviate joint inflammation mediated by local expression IL-17. We found that neutralization of CXCL1 reduced joint inflammation initially on days 3 and 5 post-IL-17 local expression, but was unable to reduce the joint swelling at later time points when arthritis was established. However, CXCL1-mediated endothelial chemotaxis in vitro was markedly reduced by anti-CXCL1 antibody (data not shown). In contrast to anti-CXCL1 treatment, anti-CXCL5 therapy effectively reduced joint inflammation, lining thickness and bone erosion throughout the disease course in the IL-17-induced arthritis model. The combination of anti-CXCL1 and CXCL5 did not ameliorate IL-17-induced joint inflammation beyond the effect observed using anti-CXCL5 alone, indicating that the clinical efficacy was due to blockade of joint CXCL5. Despite elevated levels of FGF2 in IL-17-induced arthritis model, levels of this proangiogenic factor were unaffected by anti-CXCL5 treatment indicating that the efficacy of anti-CXCL5 treatment is directly mediated through CXCR2 ligation. As demonstrated by endothelial chemotaxis data, both CXCL1 and CXCL5 bind to CXCR2, however our results suggest that ligation of these ligands may differentially activate down stream signaling pathways.

Since blockade of CXCL5, but not CXCL1, reduced IL-17 joint vascularization we next examined the mechanism by which these chemokines induce endothelial migration. Interestingly in HMVECs, CXCL1 stimulation resulted in PI3K and ERK signaling whereas only NF- $\kappa$ B pathway was activated by CXCL5 in these cells. Other studies have shown that while stimulation with CXCL1 can phosphorylate ERK1/2 pathway [42–44], activation with CXCL5 is involved with PI3K and NF- $\kappa$ B signaling pathways [45]. We further demonstrate that similar to IL-17 [23], CXCL1 mediated HMVEC migration is through PI3K activation. In contrast, inhibition of NF- $\kappa$ B suppresses endothelial chemotaxis induced by CXCL5. Perhaps inhibition of CXCL1 is ineffective in reducing joint inflammation since IL-17 is present in the mouse ankles (1,200 pg/mg and 400 pg/mg on days 4 and 10 post injection respectively [27]) and can induce angiogenesis through the same mechanism. In line with our finding others have shown that CXCL1 and 5 can differentially modulate monocyte arrest and migration [46], suggesting that ligands binding to the same receptor can have distinct functions through activating different signaling intermediates.

Reduction in joint TNF- $\alpha$  levels in the anti-CXCL5 and combination therapy may be due to the fact that IL-17-induced joint pathology is abrogated in TNF- $\alpha$  deficient mice, indicating that in this model TNF- $\alpha$  is required [3]. It has also been shown that IL-17 can directly modulate TNF- $\alpha$  secretion from macrophages [47]. Hence, suppressing IL-17-induced inflammation may reduce TNF- $\alpha$  production from macrophages in the synovial lining and sublining.

**Fig. 7** CXCL1 and CXCL5 induce endothelial migration through activating different signaling pathways. In order to determine the mechanism by which (a) CXCL1 and (c) CXCL5 activate HMVECs, cells were stimulated with these chemokines (20 ng/ml) for 0–65 min, and the cell lysates were probed for I $\kappa$ B, p-p38, p-AKT, and pERK and/or equal loading controls. To determine signaling pathways associated with b CXCL1 and d CXCL5-induced HMVEC migration, cells were treated with DMSO or inhibitors to NF- $\kappa$ B (MG-132; 1 and 5  $\mu$ M), p38 (SB203580; 1 and 5  $\mu$ M), PI3K (LY294002; 1 and 5  $\mu$ M) and ERK (PD98059; 1 and 5  $\mu$ M) 2 h in the Boyden chamber,  $n = 2$ . Values represent fold increase chemotaxis above cells migrating in response to PBS shown as mean  $\pm$  SE of two experiments in triplicate. \* Represents  $P < 0.05$



Further, both TNF- $\alpha$  and IL-17 synergize in inducing the expression of CXCL5 from RA fibroblasts. Therefore, neutralization of CXCL5 may have a negative feed back regulation on joint TNF- $\alpha$  concentrations. When RA synovial tissue fibroblasts, macrophages and HMVECs were screened for IL-17 downstream targets, CCL5 was undetected (data not shown). Therefore, reduction in joint CCL5 concentration in anti-CXCL5 and combination therapy treatment groups may be due to reduced TNF- $\alpha$  levels, since CCL5 expression is known to be modulated by TNF- $\alpha$  in RA synovial tissue fibroblasts [48, 49].

In conclusion, anti-CXCL5 treatment ameliorates IL-17-mediated arthritis by down regulating TNF- $\alpha$  and joint vascularization through an IL-17 nonoverlapping mechanism. These data support angiogenesis as an important mechanism by which IL-17 contributes to RA pathogenesis, further supporting IL-17 as a potential therapeutic target in RA.

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**Conflict of Interest** The authors declare that they have no conflict of interest.

**Ethical Standards** All experiments performed comply with the current laws of United States of America.

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# TLR5, a Novel and Unidentified Inflammatory Mediator in Rheumatoid Arthritis that Correlates with Disease Activity Score and Joint TNF- $\alpha$ Levels

Nathan D. Chamberlain,\* Olga M. Vila,\* Michael V. Volin,<sup>†</sup> Suncica Volkov,\*  
Richard M. Pope,<sup>‡</sup> William Swedler,\* Arthur M. Mandelin, II,<sup>‡</sup> and Shiva Shahrara\*

The innate immune system plays an important role in rheumatoid arthritis (RA) pathogenesis. Previous studies support the role of TLR2 and 4 in RA and experimental arthritis models; however, the regulation and pathogenic effect of TLR5 is undefined in RA. In this study, we show that TLR5 is elevated in RA and osteoarthritis ST lining and sublining macrophages and endothelial cells compared with normal individuals. Furthermore, expression of TLR5 is elevated in RA synovial fluid macrophages and RA peripheral blood monocytes compared with RA and normal peripheral blood in vitro-differentiated macrophages. We also found that TLR5 on RA monocytes is an important modulator of TNF- $\alpha$  in RA synovial fluid and that TLR5 expression on these cells strongly correlates with RA disease activity and TNF- $\alpha$  levels. Interestingly, TNF- $\alpha$  has a feedback regulation with TLR5 expression in RA monocytes, whereas expression of this receptor is regulated by IL-17 and IL-8 in RA macrophages and fibroblasts. We show that RA monocytes and macrophages are more responsive to TLR5 ligation compared with fibroblasts despite the proinflammatory response being mediated through the same signaling pathways in macrophages and fibroblasts. In conclusion, we document the potential role of TLR5 ligation in modulating transcription of TNF- $\alpha$  from RA synovial fluid and the strong correlation of TLR5 and TNF- $\alpha$  with each other and with disease activity score in RA monocytes. Our results suggest that expression of TLR5 may be a predictor for RA disease progression and that targeting TLR5 may suppress RA. *The Journal of Immunology*, 2012, 189: 475–483.

**R**heumatoid arthritis (RA) is a chronic autoimmune disorder in which the innate immune system plays an important role (1, 2). TLRs are pattern recognition receptors that are present in a number of cells and tissues, which recognize pathogen-associated molecular patterns (PAMPs) or endogenous ligands (3).

Previous studies demonstrate that expression of TLR2 and TLR4 is elevated in RA peripheral blood (PB) monocytes as well as in RA synovial fluid and synovial tissue (ST) macrophages (4–7). Increased TLR2 and TLR4 expression was detected in RA compared with osteoarthritis (OA) ST fibroblasts (8). Further, the data obtained from experimental arthritis models strongly support the role of TLR2 and TLR4 in streptococcal cell wall arthritis (9, 10) while TLR4 has been implicated in collagen-induced arthritis (11)

as well as in the IL-1RA<sup>-/-</sup> model (11, 12). However, the role of TLR5 in RA and murine models of RA is undefined.

TLR5 is expressed on a variety of cell types such as epithelial cells, neutrophils, monocytes, macrophages, and mast cells and is the receptor for the bacterial structural protein flagellin (13). Flagellin signaling via TLR5 is dependent on MyD88 and IL-1R-associated kinase 1 (14, 15) and subsequent activation of NF- $\kappa$ B, MAPK, and PI3K pathways (16–18). As with other TLR agonists, flagellin has been shown to induce dendritic cell maturation and activation (19), thereby promoting lymphocyte migration to secondary lymphoid sites (20). Others have shown that spontaneous neutrophil apoptosis is delayed by flagellin through induction of Mcl-1 and inhibition of caspase-3 (21). What remains unclear is whether TLR5 is present in RA synovium and whether ligation of this receptor plays a role in RA pathogenesis.

In this study, to our knowledge, we demonstrate for the first time that TLR5 is elevated in RA and OA ST lining and sublining macrophages and endothelial cells compared with normal (NL) controls. Consistently, our data demonstrate that TLR5 expression is greatly elevated in RA synovial fluid macrophages and PB monocytes compared with their NL counterparts. In RA monocytes, patients with higher expression of TNF- $\alpha$  expressed elevated levels of TLR5 and the concentration of both of these factors strongly correlated with increased disease activity score (DAS28). The role of TLR5 expression in RA pathogenesis was documented when the blockade of TLR5 on monocytes significantly reduced synovial fluid-mediated TNF- $\alpha$  transcription by 80%. Interestingly, we demonstrate a feedback modulation between TNF- $\alpha$  production and TLR5 ligation and expression in RA monocytes. Although in RA macrophages, TLR5 expression is induced by IL-17 and IL-8, it is significantly reduced by TLR4 ligation in both RA monocytes and macrophages. Higher expression of TLR5 was detected in RA compared with NL fibroblasts, which was upreg-

\*Division of Rheumatology, Department of Medicine, University of Illinois, Chicago, IL 60612; <sup>†</sup>Department of Microbiology and Immunology, Midwestern University, Chicago College of Osteopathic Medicine, Downers Grove, IL 60515; and <sup>‡</sup>Department of Medicine, Feinberg School of Medicine, Northwestern University, Chicago, IL 60611

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Address correspondence and reprint requests to Dr. Shiva Shahrara, Division of Rheumatology, Department of Medicine, University of Illinois, MSB 835 South Wolcott Avenue, E807-E809, Chicago, IL 60612. E-mail address: shahrara@uic.edu

Abbreviations used in this article: DAS28, disease activity score based on 28 defined joints; DMARD, disease-modifying antirheumatic drug; HSP, heat shock protein; NL, normal; OA, osteoarthritis; PAMP, pathogen-associated molecular pattern; PB, peripheral blood; poly I:C, polyinosinic-polycytidylic acid; RA, rheumatoid arthritis; ST, synovial tissue; VWF, von Willebrand factor.

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ulated by a variety of inflammatory factors excluding LPS. Hence, our data demonstrate the expression of TLR5 in RA and further documents its importance in RA disease activity and TNF- $\alpha$  modulation.

## Materials and Methods

### *Abs and immunohistochemistry*

The studies were approved by the Institutional Review Board, and all donors gave informed written consent. Because the RA ST are recruited from the practices of orthopedic surgeons these samples are de-identified; therefore the disease severity and the treatment information is unavailable. RA, OA, and NL ST were formalin fixed, paraffin embedded, and sectioned in the pathology core facility. ST were immunoperoxidase-stained using Vector Elite ABC kits (Vector Laboratories, Burlingame, CA), with diaminobenzidine (Vector Laboratories) as a chromogen. Briefly, slides were deparaffinized in xylene for 15 min at room temperature, followed by rehydration by transfer through graded alcohols. Ags were unmasked by incubating slides in proteinase K digestion buffer (DakoCytomation, Carpinteria, CA) for 10 min at room temperature. Tissues were incubated with Abs to human TLR5 (1:50; Santa Cruz Biotechnology, Santa Cruz, CA) or an IgG control Ab (Beckman Coulter, Brea, CA). Slides were evaluated by blinded observers (22–25) (A.M.M. and M.V.V.). Tissue sections were scored for lining, sublining macrophages and endothelial cell staining on a 0–5 scale (26, 27). Scored data were pooled, and the mean  $\pm$  SEM was calculated in each data group. To demonstrate location of TLR5 in RA ST serial tissue sections were stained with anti-TLR5 (1:50; Santa Cruz Biotechnology), anti-CD68 (1:100; Vector Laboratories), and anti-von Willebrand factor (VWF) (1:1000; Vector Laboratories) Abs. To localize TLR5 to macrophages in RA ST, slides were deparaffinized and unmasked as mentioned above. Using an Invision G2 kit (DakoCytomation), RA ST were stained with anti-TLR5 Ab (1:50 dilution; Santa Cruz Biotechnology) using diaminobenzidine (brown staining) as a chromogen. Thereafter, tissues were blocked (double staining blocker included in the Invision G2 kit) and stained with anti-CD68 Ab (1:100 dilution; DakoCytomation) using Fast red (red staining) as a chromogen following the manufacturers' instructions (DakoCytomation).

### *RA patient population*

RA specimens were obtained from patients with RA, diagnosed according to the 1987 revised criteria of the American College of Rheumatology (28). PB was obtained from 44 women and 4 men (mean age,  $53.7 \pm 2.7$  y). At the time of treatment, patients were receiving no treatment ( $n = 7$ ), taking nonbiological disease-modifying antirheumatic drugs (DMARDs) (methotrexate, leflunomide, and sulfasalazine azathioprine) alone ( $n = 5$ ), taking DMARDs plus hydroxychloroquine ( $n = 9$ ), taking DMARDs plus prednisone ( $n = 5$ ), taking DMARDs plus rituximab ( $n = 3$ ), taking DMARDs plus hydroxychloroquine plus minocycline ( $n = 2$ ), or taking a TNF- $\alpha$  inhibitor alone ( $n = 6$ ), with a DMARD ( $n = 8$ ), a DMARD plus prednisone ( $n = 1$ ), or with a DMARD plus hydroxychloroquine and prednisone ( $n = 2$ ). These studies were approved by the University of Illinois at Chicago Institutional Ethics Review Board, and all donors gave informed written consent. Maximum number of patients was 48; however, please refer to the figure legends for exact number of patients in each experiment.

### *Cell isolation, culture, and procedures*

NL and RA PB and RA synovial fluid mononuclear cells were isolated by Histopaque gradient centrifugation (Sigma-Aldrich, St. Louis, MO) as described previously (29, 30). Monocytes/macrophages were isolated from NL and RA PB or RA synovial fluid using a negative selection kit (StemCell Technologies, Vancouver, BC, Canada), according to the manufacturer's instructions (26, 27). Monocytes were subsequently differentiated to macrophages by culturing in RPMI 1640 medium containing 20% FBS for 7 d.

### *Quantification of chemokines and cytokines*

Human TNF- $\alpha$ , IL-6, and CCL2 (R&D Systems, Minneapolis, MN) ELISA kits were used according to the manufacturer's instructions.

### *Isolation of RA ST fibroblasts*

ST fibroblasts were isolated from fresh RA ST by mincing and digestion in a solution of dispase, collagenase, and DNase (30). Cells were used

between passages 3 and 9 and cultured in DMEM containing 10% FBS, and cell purity was validated by CD90 staining.

### *Cell treatment*

RA PB monocytes and in vitro-differentiated macrophages or RA ST fibroblasts were treated with polyinosinic-polycytidylic acid (poly I:C) (10 ng/ml, only in RA monocytes; InvivoGen, San Diego, CA), LPS (10 ng/ml; Sigma-Aldrich), IL-1 $\beta$  (10 ng/ml; R&D Systems), TNF- $\alpha$  (10 ng/ml; R&D Systems), IL-17 (50 ng/ml; R&D Systems), IL-6 (10 ng/ml; R&D Systems), IL-8 (10 ng/ml; R&D Systems), or RA synovial fluid (10%). Cells were harvested after 6 h, and the TLR5 mRNA levels were quantified by real-time RT-PCR. RA ST fibroblasts, RA PB monocytes, and differentiated macrophages were treated with flagellin Ultra pure (10 and 100 ng/ml) (endotoxin levels < 50 EU/mg) (InvivoGen), cells (6 h; for real-time RT-PCR) or conditioned media (24 h; for ELISA) were harvested following treatment, and TNF- $\alpha$ , IL-6, and CCL2 mRNA production was quantified. In a different experiment, RA monocytes from six different patients were treated with anti-TLR5 Ab or IgG (10  $\mu$ g/ml; InvivoGen) for 1 h prior to being treated with RA synovial fluid (10%;  $n = 6$ ) for 6 h. To demonstrate that reduction of RA synovial fluid-mediated TNF- $\alpha$  levels are due to blockade of TLR5 and not to the necrotic effect of this Ab in RA monocytes, cells were pretreated with anti-TLR5 Ab or IgG control 1 h prior to treating the cells with PBS or flagellin for 6 h. Subsequently, the TNF- $\alpha$  mRNA levels were quantified by real-time RT-PCR for experiments performed for Fig. 3A and 3B.

### *Real-time RT-PCR*

Total cellular RNA was extracted using TRIzol (Invitrogen, Carlsbad, CA) from the different cell types. Subsequently, reverse transcription and real-time RT-PCR were performed to determine TLR5, TNF- $\alpha$ , IL-6, and CCL2 expression levels as described previously (29–31). Relative gene expression was determined by the  $\Delta\Delta C_t$  method, and results were expressed as fold increase above conditions indicated in the figure legends.

### *Flow cytometry*

To determine TLR5<sup>+</sup> cells, NL and RA monocytes and differentiated macrophages were washed with FACS buffer (5% FBS in PBS). Thereafter, cells were blocked with 50% human serum and 0.5% BSA. Cells were then stained for PE-conjugated anti-TLR5 (Imgenex, San Diego, CA) and FITC-labeled anti-CD14 (BD Immunocytometry, Franklin Lakes, NJ) or isotype control Abs (BD Pharmingen). Percent TLR5<sup>+</sup> cells were identified as those that were CD14<sup>+</sup>TLR5<sup>+</sup>. Because of limited access to RA synovial fluid macrophages, these cells were not included in the FACS analysis.

### *Flagellin signaling pathways in RA macrophages or RA fibroblasts*

RA ST fibroblasts and macrophages ( $2 \times 10^6$ /ml) were untreated or treated with flagellin (100 ng/ml) for 0–65 min. Cell lysates were examined by Western blot analysis (30). Blots were probed with p-ERK, p-p38 MAPK, p-AKT1, p-JNK (1:1000 dilution; Cell Signaling Technology), or degradation of I $\kappa$ B (1:3000 dilution; Sigma-Aldrich) overnight or probed with ERK, p38, AKT, and JNK or actin (1:3000 dilution; Cell Signaling Technology or Sigma-Aldrich).

### *Inhibition of the signaling pathways in RA ST fibroblasts and macrophages*

To define which signaling pathways mediate flagellin-induced CCL2 secretion, RA macrophages and fibroblasts were incubated with DMSO or 10  $\mu$ M inhibitors to p38 (SB203580), ERK (PD98059), JNK (SP600125), PI3K (LY294002), or NF- $\kappa$ B (MG-132) for 1 h in RA-differentiated macrophages or fibroblasts. Cells were subsequently activated with flagellin (100 ng/ml) for 24 h, and the media were collected to quantify the levels of CCL2 using ELISA.

### *Statistical analysis*

The data were analyzed using one-way ANOVA, followed by a post hoc two-tailed Student *t* test for paired and unpaired samples. In RA monocytes, TLR5 and TNF- $\alpha$  mRNA expression was correlated with each other using the  $\Delta\Delta C_t$  method. Further TLR5 or TNF- $\alpha$  mRNA mass was normalized to its GAPDH mass, and values were correlated with DAS28 score using linear regression analysis in RA monocytes. The *p* values < 0.05 were considered significant.

## Results

### TLR5 elevated in RA and OA ST

To characterize the expression pattern of TLR5 in RA and OA compared with controls, ST were stained with Abs against TLR5. We found that both in RA and OA, TLR5 immunostaining was significantly higher on ST lining and sublining macrophages and endothelial cells compared with NL ST (Fig. 1A–D). Consistently, TLR5 staining was colocalized to RA ST CD68<sup>+</sup> (Fig. 1E, 1F, 1H) and VWF<sup>+</sup> cells (Fig. 1E, 1G). Although previous studies demonstrate that fibroblasts in the lining and macrophages in the lining and sublining express TLR2 and 4 (32), expression of these receptors has not been reported in endothelial cells. Therefore, TLR5 may be a member of the TLR family that is uniquely elevated on RA and OA endothelial cells.

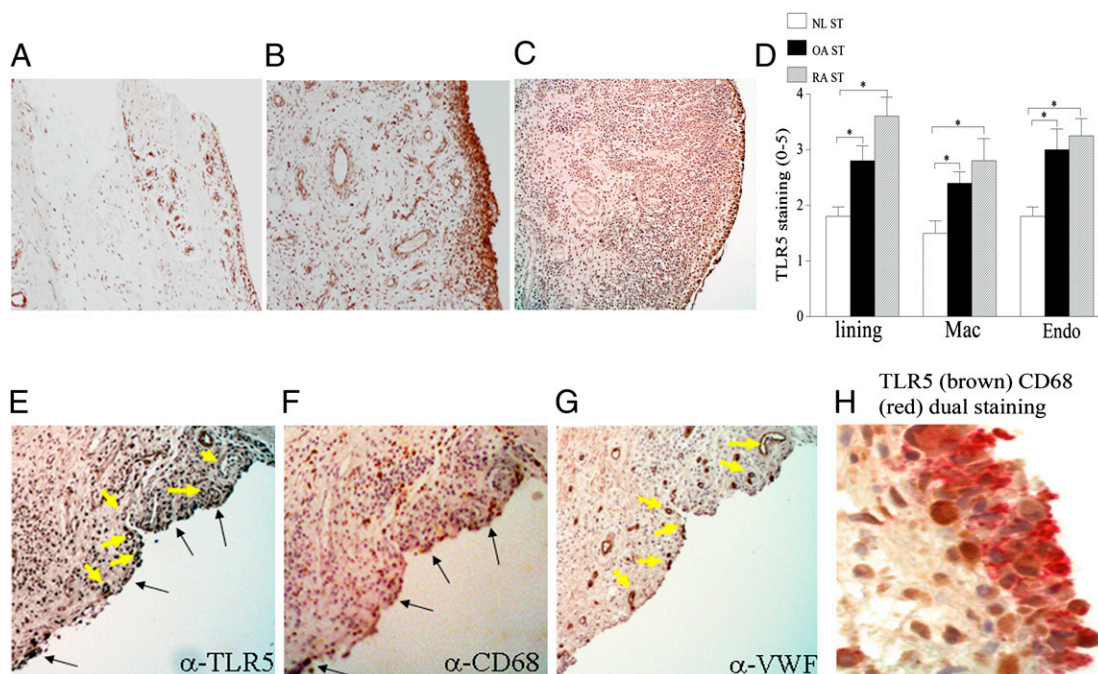
### RA synovial fluid macrophages and RA PB monocytes express upregulated levels of TLR5

Because TLR5 immunostaining was elevated in RA ST lining and sublining macrophages, we asked whether mRNA and/or cell surface expression of this receptor was increased in RA synovial fluid macrophages compared with RA and NL PB monocytes or differentiated macrophages. Using microarray analysis, TLR5 was identified as one of the genes (3.2-fold higher;  $p = 1.58 \times 10^{-10}$ ) that was greatly increased in RA synovial fluid macrophages compared with NL macrophages. These results were confirmed when real-time RT-PCR demonstrated that the expression of TLR5 was elevated 9- and 35-fold in RA synovial fluid macrophages compared with RA and normal PB-differentiated macrophages, respectively (Fig. 2A). Furthermore, levels of TLR5 were 7- and 3-fold greater in RA PB monocytes compared with RA PB-differentiated macrophages and normal monocytes (Fig. 2A). Consistent with our mRNA results, FACS

analysis demonstrated that percent TLR5 was significantly reduced both in RA and NLs when monocytes were differentiated into macrophages; however, percent TLR5 was 4-fold higher in RA monocytes and macrophages compared with NL counterpart cells (Fig. 2B, 2C). Despite reduction of TLR5 expression during monocyte to macrophage differentiation, TLR5 expression is significantly increased in macrophages isolated from RA joints, compared with control or RA PB macrophages. Altogether, our results suggest that RA ST and fluid macrophages as well as RA PB monocytes may be an important source for TLR5 response.

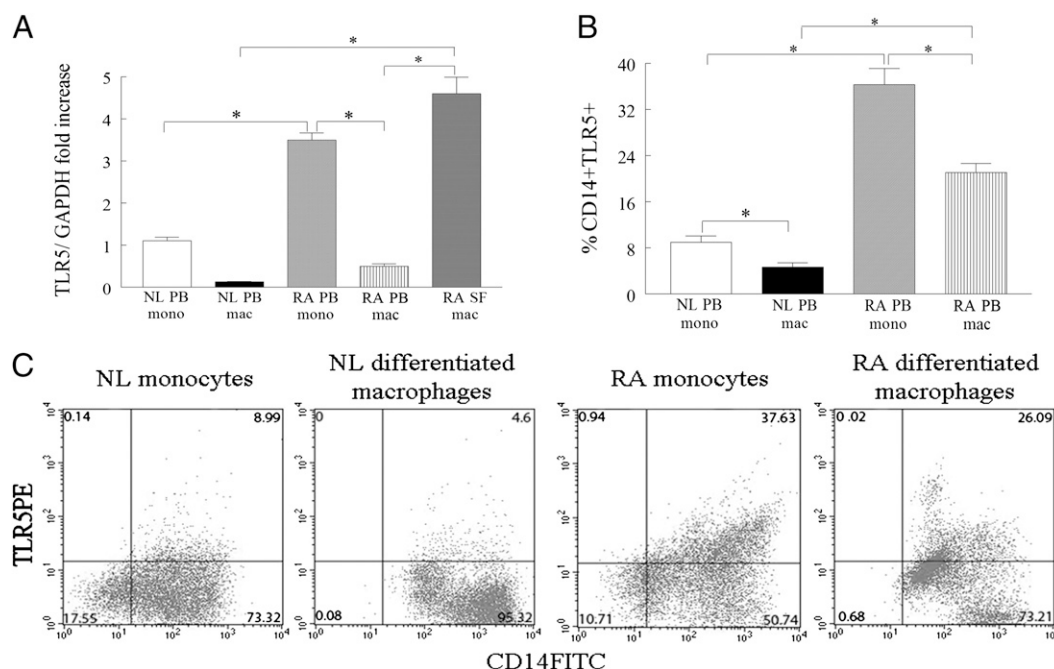
### TLR5 ligation can modulate synovial fluid-induced TNF- $\alpha$ transcription in RA monocytes and expression of TLR5 on these cells strongly correlates with DAS28 and TNF- $\alpha$

Because expression of TLR5 was higher in RA PB monocytes compared with differentiated macrophages, we asked whether ligation of TLR5 in RA monocytes may affect disease pathogenesis. Previous studies have identified a number of endogenous TLR2 and/or TLR4 ligands in RA synovial fluid (33, 34). Hence, synovial fluid-mediated TNF- $\alpha$  transcription in RA monocytes was examined to determine whether endogenous TLR5 ligand(s) were present in RA synovial fluid. Our results show that blockade of TLR5 on RA monocytes greatly downregulates (5-fold decrease; 80% reduction) TNF- $\alpha$  transcription activated by RA synovial fluid (Fig. 3A), suggesting that ligation of TLR5 by potential endogenous ligands expressed in RA synovial fluid may be partially responsible for joint TNF- $\alpha$  modulation. We further validated that the inhibitory effect of anti-TLR5 Ab on RA synovial fluid-mediated TNF- $\alpha$  was specifically due to blockade of TLR5 ligation and had no effect on cell necrosis (Fig. 3B). Given that ligation of TLR5 plays a role in joint TNF- $\alpha$  regulation, we asked whether expression of these two factors correlate with each other



**FIGURE 1.** TLR5 expression is increased in RA and OA ST lining and sublining macrophages and endothelial cells compared with NL ST. NL (A), OA (B), and RA (C) ST were stained with anti-human TLR5 (A–C) (original magnification  $\times 200$ ), and positive immunostaining was scored on a 0–5 scale (D). ST lining and sublining macrophage (Mac) and endothelial (Endo) immunostaining are shown as mean  $\pm$  SEM; ( $n = 5–7$ ).  $*p < 0.05$ . RA serial sections were stained with anti-TLR5 (E), anti-CD68 (F), and anti-VWF (G) Abs to distinguish TLR5 immunostaining on RA ST macrophages and endothelial cells (original magnification  $\times 400$ ). Black (E, F) and yellow arrows (E, G) demonstrate colocalization of TLR5 on CD68<sup>+</sup> and VWF<sup>+</sup> cells. (H) RA ST were stained for anti-TLR5 (brown staining) and anti-CD68 Abs (fast red staining) (original magnification  $\times 800$ ) to demonstrate TLR5 costaining on lining macrophages.

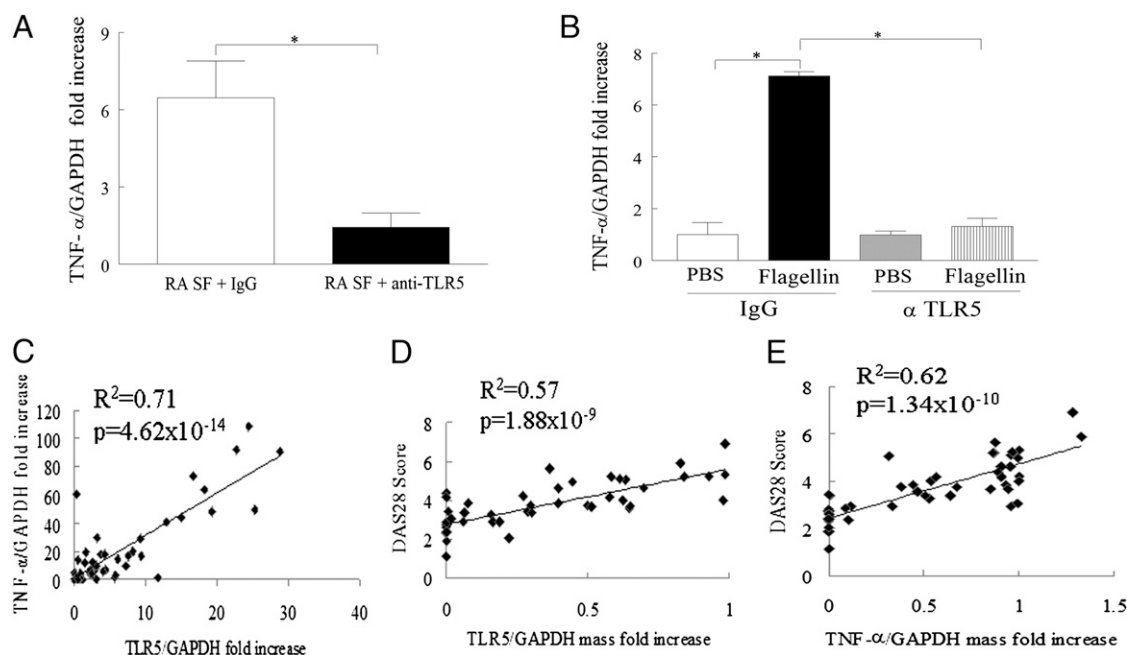




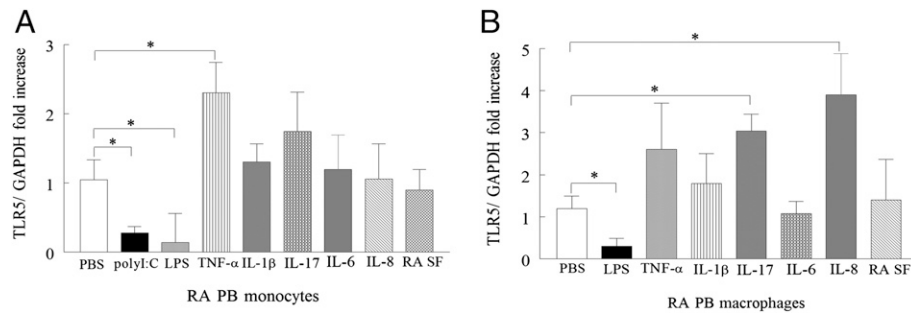
**FIGURE 2.** TLR5 is upregulated in RA synovial fluid (SF) compared with RA and NL PB macrophages. **(A)** TLR5 mRNA levels were determined in NL ( $n$  for monocytes [mono] or macrophages [mac] = 11 or 18) and RA PB monocytes ( $n$  = 11) and differentiated macrophages ( $n$  = 15) as well as in RA SF macrophages ( $n$  = 10) by using real-time RT-PCR. The data are shown as fold increase above NL PB monocytes and are normalized to GAPDH. **(B)** Normal and RA PB monocytes and differentiated macrophages were immunostained with CD14 labeled with FITC and TLR5 conjugated with PE to determine percent TLR5<sup>+</sup> cells ( $n$  = 6–10). The values are presented as mean  $\pm$  SEM of percent CD14<sup>+</sup>TLR5<sup>+</sup> in each cell population. **(C)** Representative flow cytometry histograms showing CD14<sup>+</sup>TLR5<sup>+</sup> in NL and RA PB monocytes and differentiated macrophages. \* $p$  < 0.05.

and/or DAS28. We found that the levels of TLR5 and TNF- $\alpha$  in RA monocytes were closely related ( $R^2 = 0.71$ ;  $p = 4.62 \times 10^{-14}$ ) (Fig. 3C). Furthermore, data analyzed by regression analysis

demonstrated that patients with greater levels of DAS28 had increased expression of TLR5 ( $R^2 = 0.57$ ;  $p = 1.88 \times 10^{-9}$ ) (Fig. 3D) and TNF- $\alpha$  ( $R^2 = 0.62$ ;  $p = 1.34 \times 10^{-10}$ ) (Fig. 3E) in RA



**FIGURE 3.** TLR5 ligation can regulate synovial fluid (SF)-induced TNF- $\alpha$  transcription in RA monocytes and expression of TLR5 on these cells strongly correlates with DAS28 and TNF- $\alpha$  levels. **(A)** RA monocyte from six different patients were treated with anti-TLR5 Ab or IgG (10  $\mu$ g/ml; InvivoGen) for 1 h prior to being treated with RA SF (10%;  $n$  = 6) for 6 h. **(B)** RA monocytes were pretreated with anti-TLR5 Ab or IgG control 1 h prior to treating the cells with PBS or flagellin for 6 h. Subsequently, the TNF- $\alpha$  mRNA levels were quantified in (A) and (B) by real-time RT-PCR and normalized to GAPDH value. In (A), the data are shown as fold increase above RA monocytes treated with RA SF plus anti-TLR5 Ab. Whereas in (B), the data are shown as fold increase above the IgG-pretreated PBS group. Linear regression analysis was used to compare TNF- $\alpha$  levels with TLR5 ( $n$  = 48 RA patients) as well as DAS28 score with expression of TLR5 (RNA mass normalized to GAPDH mass) **(D)** ( $n$  = 45 RA patients) or TNF- $\alpha$  (RNA mass normalized to GAPDH mass) **(E)** ( $n$  = 45 RA patients) in RA monocytes. The mRNA expression in RA monocytes is shown as a fold increase above NL PB monocytes and is normalized to GAPDH. \* $p$  < 0.05.



**FIGURE 4.** Proinflammatory factors induce the expression of TLR5 in RA PB monocytes and in vitro-differentiated macrophages. RA PB monocytes (**A**) or in vitro-differentiated macrophages (**B**) were untreated (PBS) or treated with poly I:C (10 ng/ml; only in RA monocytes) LPS (10 ng/ml), IL-1 $\beta$  (10 ng/ml), TNF- $\alpha$  (10 ng/ml), IL-17 (50 ng/ml), IL-6 (10 ng/ml), IL-8 (10 ng/ml), or RA synovial fluid (SF) (10%) for 6 h, and expression of TLR5 was measured by real-time RT-PCR ( $n = 5-12$ ). The data are shown as fold increase above untreated RA PB monocytes or macrophages and are normalized to GAPDH. Values demonstrate mean  $\pm$  SEM. \* $p < 0.05$ .

monocytes. These results suggest that RA disease expression is related to ligation of TLR5 and production of TNF- $\alpha$  from RA monocytes.

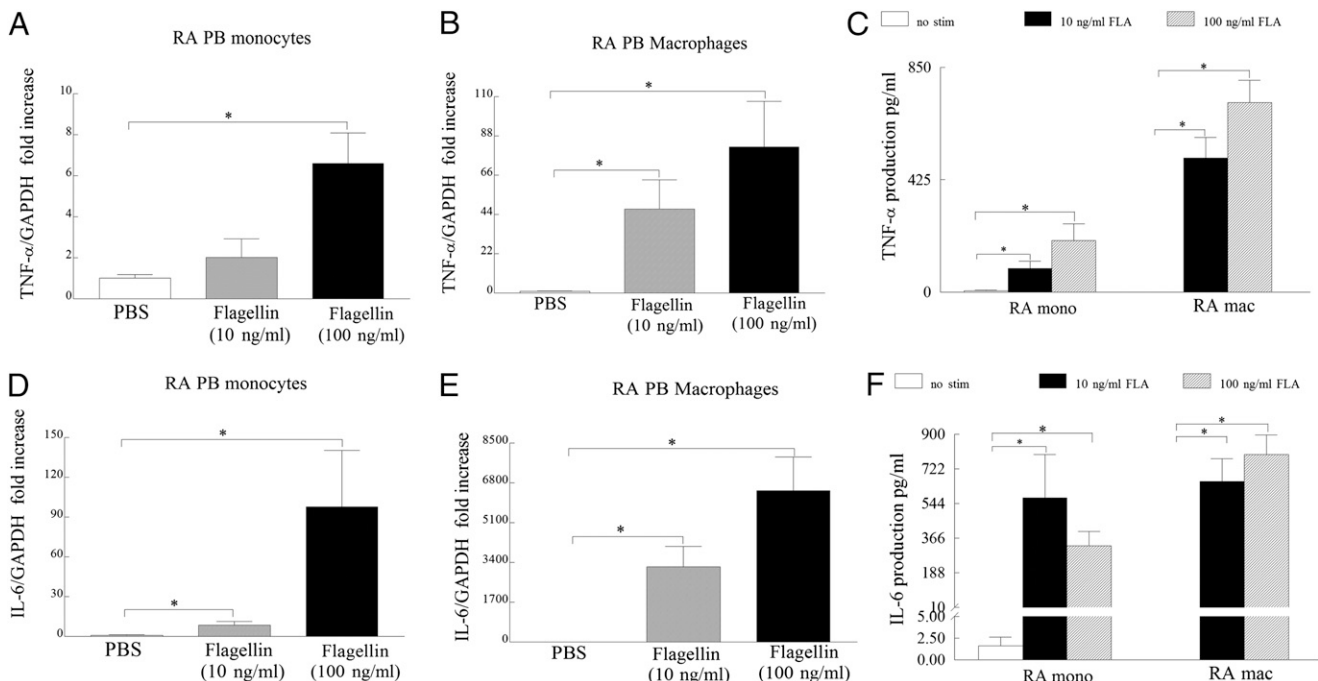
#### *Proinflammatory factors regulate expression of TLR5 in RA monocytes and macrophages*

To determine which factors modulate expression of TLR5 in RA PB monocytes or in vitro-differentiated macrophages, cells were either untreated or treated with poly I:C (only in RA monocytes), LPS, IL-1 $\beta$ , TNF- $\alpha$ , IL-17, IL-6, IL-8, or RA synovial fluid. Results from these experiments demonstrate TLR5 expression was modulated by TNF- $\alpha$  in RA monocytes and by IL-17 and IL-8 in RA macrophages; however, expression levels of TLR5 were suppressed by TLR3 and TLR4 ligation in RA monocytes and/or differentiated macrophages (Fig. 4). Hence, the data suggest that with the exception of LPS, the expression of TLR5 in RA

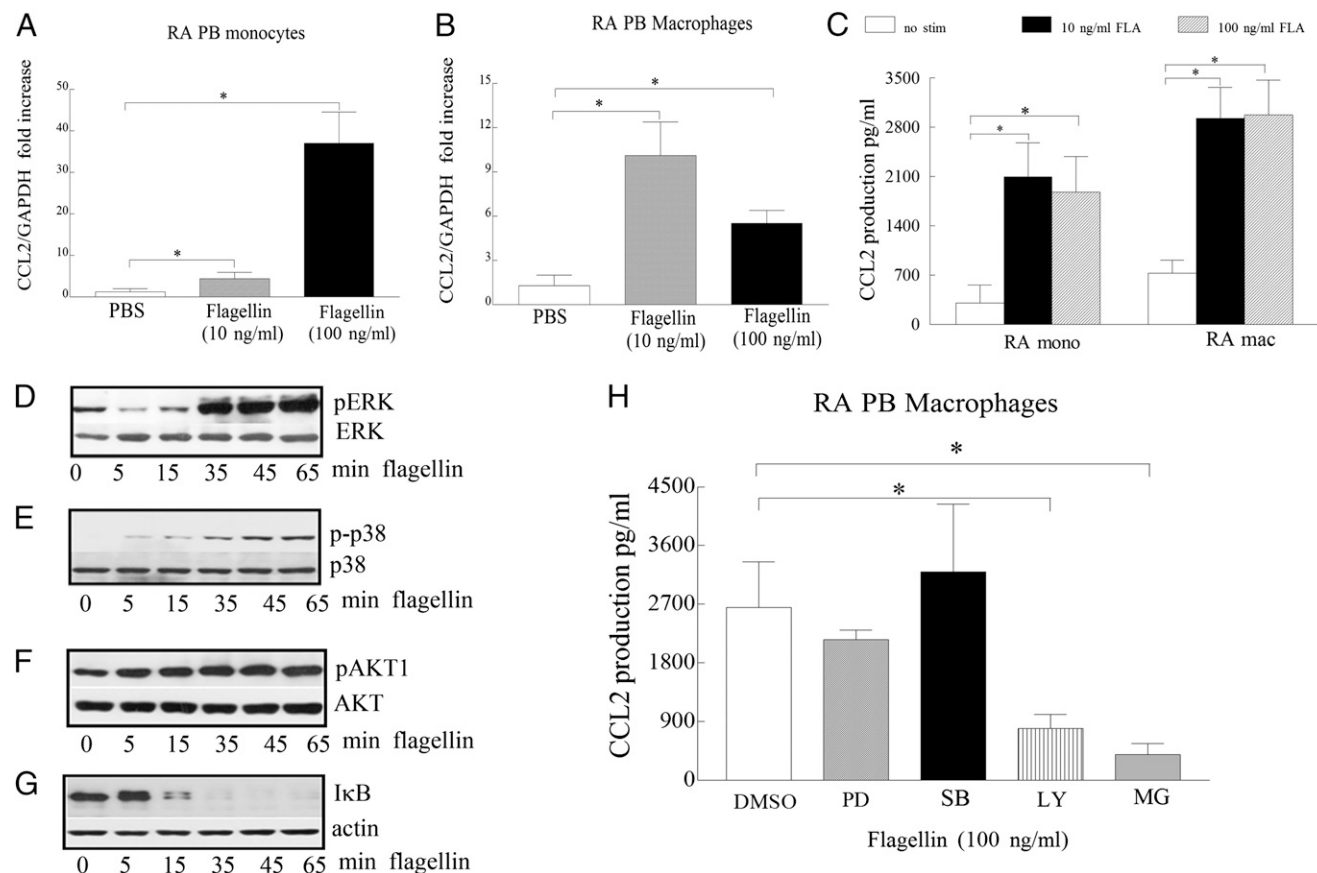
monocytes and macrophages is differentially regulated in RA monocytes and differentiated macrophages.

#### *Ligation of TLR5 induces production of proinflammatory factors in RA PB monocytes and macrophages*

Next, we asked whether RA monocytes and differentiated macrophages respond to ligation of flagellin to TLR5. For this purpose, RA monocytes and differentiated macrophages were activated with different doses of flagellin, and cells were screened for transcription (6 h) and production (24 h) of proinflammatory factors such as TNF- $\alpha$ , IL-6, and CCL2. Generally, transcription but not the secretion of TNF- $\alpha$ , IL-6, and CCL2 was dose dependently increased with flagellin stimulation in RA monocytes and differentiated macrophages (Figs. 5, 6A–C). Although TLR5 expression was greatly elevated in RA monocytes compared with RA-differentiated macrophages, TLR5 ligation resulted in higher



**FIGURE 5.** TLR5 ligation induces expression and production of TNF- $\alpha$  and IL-6 in RA monocytes and macrophages. RA monocytes (**A**, **D**) and differentiated macrophages (**B**, **E**) were either untreated (PBS) or treated with flagellin at 10 or 100 ng/ml for 6 h, and expression levels of TNF- $\alpha$  (**A**, **B**) and IL-6 (**D**, **E**) were quantified by real-time RT-PCR,  $n = 6-10$ . The data are shown as fold increase above untreated cells and were normalized to GAPDH values. Supernatants were harvested from RA monocytes (mono) or differentiated macrophages (mac) untreated (PBS) or treated with flagellin 10 or 100 ng/ml for 24 h, and TNF- $\alpha$  (**C**) and IL-6 (**F**) levels were determined by ELISA;  $n = 5-8$ . Values demonstrate mean  $\pm$  SEM. \* $p < 0.05$ .



**FIGURE 6.** CCL2 levels are increased following TLR5 ligation in RA monocytes and macrophages. Furthermore, in RA macrophages, flagellin-induced CCL2 production is modulated by PI3K and NF- $\kappa$ B pathways. RA monocytes (**A**) and differentiated macrophages (**B**) were either untreated (PBS) or treated with flagellin at 10 or 100 ng/ml for 6 h, and expression levels of CCL2 (**A**, **B**) were quantified by real-time RT-PCR;  $n = 6-10$ . The data are shown as fold increase above untreated cells and were normalized to GAPDH. Supernatants were harvested from RA monocytes (mono) or differentiated macrophages (mac) untreated (PBS) or treated with flagellin at 10 or 100 ng/ml for 24 h, and CCL2 (**C**) levels were determined by ELISA. Values are the mean  $\pm$  SEM;  $n = 5-8$ . To determine the mechanism of TLR5 activation in RA macrophages, cells were stimulated with flagellin at 100 ng/ml for 0–65 min, and the cell lysates were probed for, p-ERK (**D**), p-p38 (**E**), p-AKT1 (**F**), and degradation of I $\kappa$ B (**G**) and/or equal loading control. To examine which of the signaling pathways were associated with TLR5-induced CCL2 production, in RA macrophages, cells were untreated (DMSO) or treated with 10  $\mu$ M inhibitors to ERK (PD98059), p38 (SB203580), PI3K (LY294002), or NF- $\kappa$ B (MG-132) for 1 h. Cells were subsequently activated with flagellin (100 ng/ml) for 24 h, and the conditioned media were collected to quantify the levels of CCL2 using ELISA (**H**). Values are the mean  $\pm$  SEM;  $n = 4$ . \* $p < 0.05$ .

(TNF- $\alpha$ ) or comparable production of proinflammatory factors (IL-6 and CCL2) in RA macrophages compared with that of monocytes. These results suggest that despite lower expression of TLR5 in RA macrophages, both monocytes and macrophages respond comparably to TLR5 ligation.

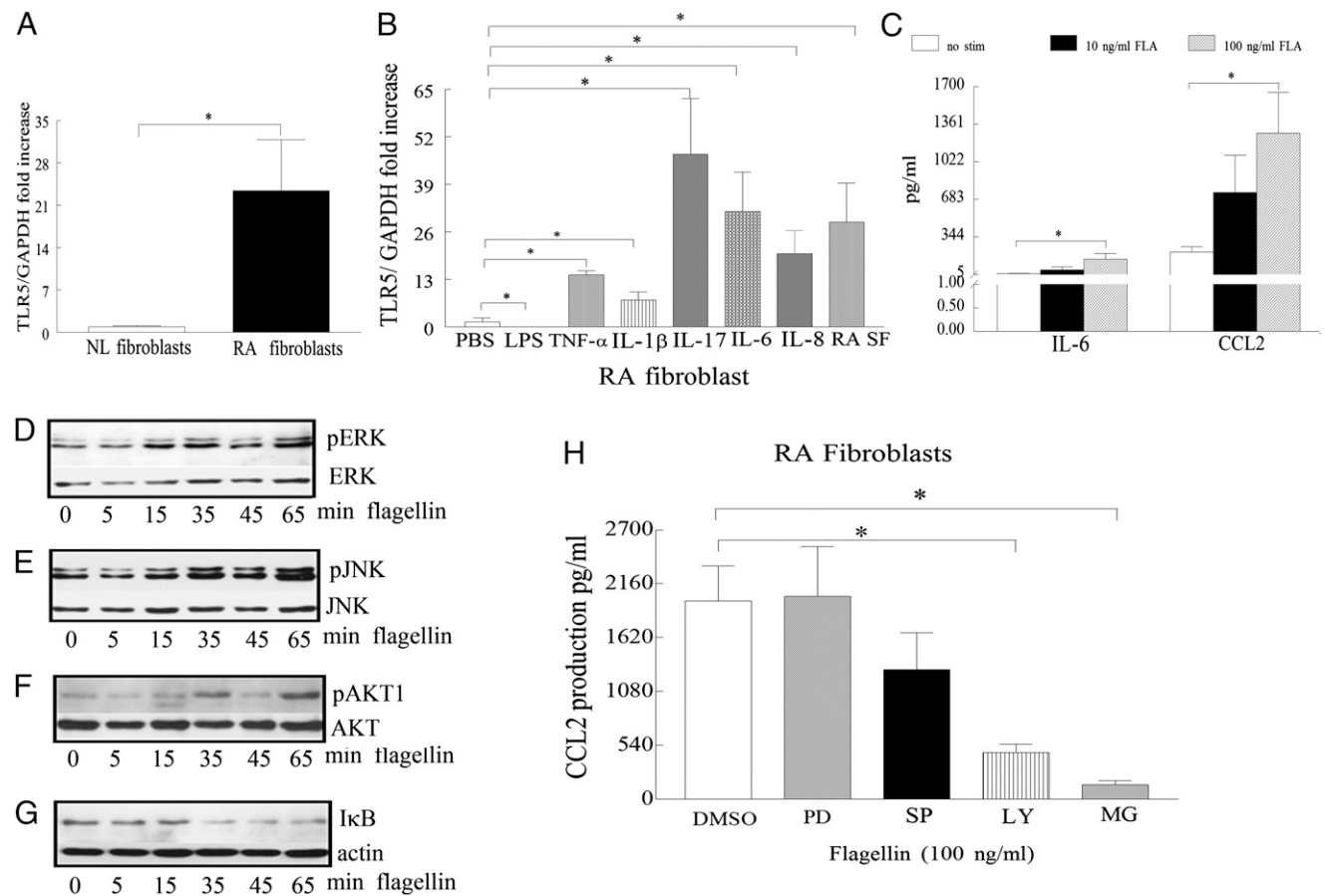
#### Flagellin-induced CCL2 is regulated by NF- $\kappa$ B and PI3K pathways in RA macrophages

We next inhibited flagellin-activated pathways in RA-differentiated macrophages to determine signaling pathways contributing to flagellin-mediated proinflammatory factor production. We found that p38 (5 min), AKT1 (5 min), ERK (35 min), and NF- $\kappa$ B (15 min) pathways (Fig. 6D–G) were activated by flagellin stimulation in RA-differentiated macrophages. We chose to examine the regulation of flagellin-induced CCL2 because this chemokine was detected both in RA-differentiated macrophages and fibroblasts. Although chemical inhibitors to NF- $\kappa$ B and PI3K suppressed flagellin-induced CCL2 secretion by 3- to 6-fold ( $p < 0.05$ ; Fig. 6H), inhibition of p38 or ERK pathway did not reduce the levels of CCL2 secretion by RA-differentiated macrophages. Our results suggest that activation of NF- $\kappa$ B and PI3K by flagellin regulates CCL2 production in RA-differentiated macrophages.

#### TLR5 is elevated in RA fibroblasts and its expression is responsive to stimulation

On the basis of our histological data, we asked whether expression of TLR5 was elevated in RA compared with NL ST fibroblasts. Results obtained from real-time RT-PCR demonstrate that TLR5 (Fig. 7A) expression was 23-fold greater in RA compared with NL ST fibroblasts. We next show that with the exception of LPS, all other proinflammatory factors such as TNF- $\alpha$  (14-fold), IL-1 $\beta$  (7-fold), IL-17 (47-fold), IL-6 (31-fold), IL-8 (20-fold), and RA synovial fluid (28-fold) greatly upregulate the expression of TLR5 in RA fibroblasts (Fig. 7B). To determine whether RA fibroblasts respond to TLR5 ligation, flagellin-activated cells (at two different doses) were screened for a variety of proinflammatory factors. Unlike RA monocytes and differentiated macrophages that are very responsive to flagellin stimulation, RA fibroblasts produce increased levels of IL-6 and CCL2 only when activated with a higher dose of flagellin (100 ng/ml). We found that flagellin activates JNK (15min), ERK (15 min), AKT1 (35min), and NF- $\kappa$ B (35 min) pathways in RA fibroblasts (Fig. 7D–G). Both in RA fibroblasts and macrophages, ligation of TLR5-induced CCL2 production was modulated by NF- $\kappa$ B and PI3K activation (Figs. 6H, 7H). These data suggest that although





**FIGURE 7.** Expression of TLR5 in RA ST fibroblasts is very responsive to stimulation; however, only higher concentrations of flagellin can induce production of IL-6 and CCL2, which is modulated by PI3K and NF- $\kappa$ B pathways. **(A)** TLR5 mRNA levels were determined in NL and RA ST fibroblasts using real-time RT-PCR ( $n = 7$ ). The data are shown as fold increase above NL ST fibroblasts and are normalized to GAPDH. **(B)** RA ST fibroblasts were untreated (PBS) or treated with LPS (10 ng/ml), IL-1 $\beta$  (10 ng/ml), TNF- $\alpha$  (10 ng/ml), IL-17 (50 ng/ml), IL-6 (10 ng/ml), IL-8 (10 ng/ml), or RA SF (10%) for 6 h, and the expression of TLR5 was measured by real-time RT-PCR ( $n = 5-12$ ). The data are shown as fold increase above untreated RA fibroblasts and are normalized to GAPDH. **(C)** Supernatants were harvested from RA fibroblasts untreated (PBS) or treated with flagellin at 10 or 100 ng/ml for 24 h, and IL-6 and CCL2 levels were determined by ELISA ( $n = 4$ ). Values demonstrate mean  $\pm$  SEM; \* $p < 0.05$ . To determine the mechanism of TLR5 activation in RA fibroblasts, cells were stimulated with flagellin 100 ng/ml for 0–65 min, and the cell lysates were probed for p-ERK **(D)**, p-JNK **(E)**, p-AKT1 **(F)**, and degradation of I $\kappa$ B **(G)** and/or equal loading control. To examine which of the signaling pathways were associated with TLR5-induced CCL2 production, in RA fibroblasts, cells were untreated (DMSO) or treated with 10  $\mu$ M inhibitors to ERK (PD98059), JNK (SP600125), PI3K (LY294002), or NF- $\kappa$ B (MG-132) for 1 h. Cells were subsequently activated with flagellin (100 ng/ml) for 24 h, and the conditioned media were collected to quantify the levels of CCL2 using ELISA **(H)**. Values are the mean  $\pm$  SEM;  $n = 4$ . \* $p < 0.05$ .

ligation of TLR5 can induce production of proinflammatory factors through the same signaling pathways in both RA macrophages and fibroblasts, macrophages are comparatively more sensitive to TLR5 activation.

## Discussion

In the current study, we show that RA and OA ST lining and sublining macrophages and endothelial cells express higher levels of TLR5 than tissues of normal controls. We found that transcription levels of TLR5 were elevated in RA synovial fluid macrophages and RA monocytes compared with RA and NL-differentiated macrophages. Confirming histological studies, TLR5 levels were also elevated in RA compared with NL fibroblasts. We show that in RA fibroblasts and macrophages, the TLR5 mRNA concentration was modulated by IL-17 and IL-8. Despite elevated cell surface levels of TLR5 in RA PB monocytes compared with differentiated macrophages, production of proinflammatory factors was comparable in both cell types, which was higher than what was secreted by RA fibroblasts following ligation. Most importantly, we document that in RA monocytes, TLR5 is

a regulator of synovial fluid-mediated TNF- $\alpha$  transcription, and levels of this receptor are strongly correlated to TNF- $\alpha$  and DAS28 score. These results suggest that TLR5 endogenous ligand(s) in the RA joint may potentially activate TLR5<sup>+</sup> RA monocytes and contribute to production of joint TNF- $\alpha$  and perpetuation of disease activity.

To our knowledge, we show for the first time that TLR5 expression is elevated in RA and OA ST lining and sublining macrophages and endothelial cells compared with normal individuals. However, expression of TLR5 has not been associated with systemic lupus erythematosus (35). Previous studies demonstrate that TLR5 is expressed in dendritic cells (36), neutrophils (37), and synovial fibroblasts from patients with juvenile idiopathic arthritis (38) and in a number of endothelial cell lines (39) however its expression is undefined in RA ST and blood cells.

Interestingly, we found that differentiation of RA monocytes to macrophages reduces TLR5 expression, as confirmed by both real-time RT-PCR and FACS studies. The same trend was also observed in normal cells. As with TLR5, expression of TLR2 was greater in normal monocytes compared with PB-differentiated macrophages,

whereas similar levels of TLR4 were detected in normal PB monocytes and differentiated macrophages (7). Furthermore, elevated expression levels of TLR2 and TLR4 in RA synovial fluid macrophages compared with normal macrophages (7) is consistent with our findings with TLR5. In contrast to our results, others have shown that TLR5 is similarly expressed in normal PB monocytes and macrophages (37). The discrepancy in the data may be due to monocyte isolation technique as well as using 100 ng/ml M-CSF for macrophage differentiation studies (37). In addition, we demonstrate that TLR3 and TLR4 ligation reduced TLR5 expression on RA monocytes and/or macrophages or fibroblasts. TRIF is an adaptor protein that is shown to degrade TLR5 expression through a caspase-dependent manner (40). Hence, suppression of TLR5 expression in RA cells may be due to activation of TRIF by TLR3 or TLR4 ligation. In RA fibroblasts, although expression of TLR5 is reduced by TLR4 ligation, stimulation with IL-1 $\beta$  has a reverse effect, and this may be due to its lack of association with the TRIF pathway (41). With the exception of LPS, TLR5 expression is differentially regulated in monocytes and macrophages. Others have shown that in human monocytes, expression of TLR5 is suppressed by TLR2 ligation as well as stimulation with IFN- $\gamma$  and GM-CSF; however, TLR5 expression is greatly increased by flagellin ligation (37, 42). In RA macrophages and fibroblasts, expression of TLR5 was modulated by IL-17. Previous studies have shown that TLR5 ligation can induce Th17 cell differentiation in normal PB mononuclear cells (43) as well as the production of IL-17 in splenocytes (44). IL-17 can also enhance TLR5-induced TNF- $\alpha$  and IL-1 $\beta$  production in epithelial cells (45). These results suggest that expression and ligation of TLR5 on cells present in RA ST lining may be in feedback regulation with Th17 cell differentiation and production of joint IL-17.

Our results suggest that TLR5 endogenous ligand(s) may be present in synovial fluid because blockade of this receptor on monocytes significantly reduces TNF- $\alpha$  transcription induced by synovial fluid. Interestingly, a number of endogenous TLR ligands have been identified in RA ST and fluid including fibrinogen, heat shock protein (HSP)60, 70, and 96 and EDA fibronectin that bind to TLR2 and/or TLR4 (33, 34). Previous studies demonstrate that TLR5-transfected reporter HEK 293T cells stimulated with full-length HSP70 had enhanced flagellin-induced NF- $\kappa$ B-mediated luciferase activity; however, this effect was not detected with HSP70 treatment alone (46). These findings suggest that HSP70 expressed in RA synovial fluid (47), ST macrophages, and fibroblasts (33) may be a chaperone protein for TLR5 endogenous ligand(s) (48). Lectins have also been identified as novel agonists for cell surface-bound TLRs (49). On the basis of earlier investigations, there is a possibility that HSPs (33, 34) and/or lectins (49) may be potential TLR5 endogenous ligands in RA joints. Therefore, studies are currently being conducted to identify RA synovial fluid TLR5 endogenous ligands (not within the scope of this study).

Our results suggest that ligation of synovial fluid TLR5 endogenous ligands to TLR5<sup>+</sup> monocytes can contribute to production of joint TNF- $\alpha$ , which in turn can further upregulate expression of TLR5 on these cells. Once RA monocytes reach their destination in the joint and differentiate to macrophages, TLR5 expression is no longer modulated by TNF- $\alpha$ , and their levels are reduced; however, they remain at least as responsive to ligation as RA monocytes. Perhaps in RA monocytes, TLR5 levels correlate with DAS28 and TNF- $\alpha$  and are in a feedback regulation with TNF- $\alpha$  by producing and responding to this factor to perpetuate disease.

When RA monocytes and differentiated macrophages were stimulated with flagellin, similar levels of IL-6 and CCL2 were

produced despite RA macrophages having lower TLR5 expression compared with RA monocytes. This may be due to monocytes being in circulation, whereas macrophages are immobilized in the inflammatory milieu of RA ST in cell-to-cell contact with other macrophages or RA fibroblasts, therefore amplifying the activation response. It is also possible that macrophages from RA ST, like those from RA synovial fluid, have higher TLR5 expression compared with RA monocytes, and maybe the presence of proinflammatory factors is required to enhance TLR5 expression during the differentiation process, which is available in the RA joint and unavailable in the culture system. In contrast to our results, other studies were unable to detect TNF- $\alpha$  production when normal monocytes were activated with flagellin (50). This may be due to lower levels of TLR5 expression in normal cells compared with RA monocytes as well as isolation and culturing methods. However, consistent with our data, they were able to demonstrate high levels of CCL2 following TLR5 ligation in normal monocytes (50).

Unlike RA fibroblasts where only higher concentrations of flagellin (100 ng/ml) are capable of inducing expression of IL-6 and CCL2, in RA monocytes and macrophages, ligation of TLR5 with lower concentrations (10 ng/ml) can produce these factors. Conversely, in skeletal muscle cells, ligation of TLR5 was unable to produce significant levels of CCL2 without IFN- $\gamma$  priming (51). Despite activation of nonoverlapping pathways in RA fibroblasts and macrophages by TLR5 ligation, CCL2 production was modulated by inhibition of NF- $\kappa$ B and PI3K in both cell types. In contrast to our results, blockade of PI3K or use of PI3K/AKT-deficient mice resulted in marked increase in flagellin-induced IL-6 or IL-8/KC levels (52), indicating that the proinflammatory factors produced as a result of TLR5 ligation are differentially regulated in mice and humans.

In conclusion, to our knowledge, we demonstrate for the first time that TLR5 is expressed in RA ST macrophages and fibroblasts as well as RA PB monocytes. We further document modulating factors and pathways contributing to TLR5 inflammatory response. Moreover, our study highlights that there is a strong correlation between TNF- $\alpha$  and TLR5 expression with disease activity in RA monocytes suggesting that TLR5 may be a TNF- $\alpha$  responsive gene that is linked to RA progression.

## Disclosures

The authors have no financial conflicts of interest.

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## Role of the CCL21 and CCR7 Pathways in Rheumatoid Arthritis Angiogenesis

Sarah R. Pickens,<sup>1</sup> Nathan D. Chamberlain,<sup>1</sup> Michael V. Volin,<sup>2</sup> Richard M. Pope,<sup>3</sup>  
Nicholas E. Talarico,<sup>3</sup> Arthur M. Mandelin II,<sup>3</sup> and Shiva Shahrara<sup>1</sup>

**Objective.** To determine the role of CCL21 and its receptor CCR7 in the pathogenesis of rheumatoid arthritis (RA).

**Methods.** Histologic studies were performed to compare the expression of CCR7 and CCL21 in RA synovial tissue. Next, the role of CCL21 and/or CCR7 in angiogenesis was examined using in vitro chemotaxis, tube formation, and in vivo Matrigel plug assays. Finally, the mechanism by which CCL21 mediates angiogenesis was determined by Western blot analysis and endothelial cell chemotaxis and tube formation assays.

**Results.** CCL21, but not CCL19, at concentrations present in the RA joint, induced human microvascular endothelial cell (HMVEC) migration that was mediated through CCR7 ligation. Suppression of the phosphatidylinositol 3-kinase pathway markedly reduced CCL21-induced HMVEC chemotaxis and tube formation; however, suppression of the ERK and JNK pathways had no effect on these processes. Neutralization of either CCL21 in RA synovial fluid or CCR7 in HMVECs significantly reduced the induction of HMVEC migration and/or tube formation by RA synovial fluid. We further demonstrated that CCL21 is angiogenic, by showing its ability to promote blood vessel growth in

Matrigel plugs in vivo at concentrations that are present in RA joints.

**Conclusion.** Angiogenesis is dependent on endothelial cell activation, migration, and proliferation, and inhibition of angiogenesis may provide a novel therapeutic approach in RA. This study identified a novel function of CCL21 as a mediator of RA angiogenesis, supporting CCL21/CCR7 as a therapeutic target in RA.

CCL21 and CCL19 are CCR7-binding chemokines that modulate the circulation of T cells and dendritic cells within lymphoid and peripheral organs (1). Consistently, CCR7-deficient mice show defective migration of lymphocytes and dendritic cells into the T cell zones (2). Previous studies have demonstrated that CCL21 is expressed by lymph node endothelial cells and functions as an endothelial cell growth factor (3). Expression of CCL21 is associated with the formation of tertiary lymphoid tissue; this process is known as lymphoid neogenesis (4). Recent studies showed reduced inflammation in CCR7-deficient mice with antigen-induced arthritis (5); it is unclear whether amelioration of arthritis was attributable to the abnormalities in lymphoid architecture detected in CCR7<sup>-/-</sup> mice.

A role for CCR7 and/or CCL21 in rheumatoid arthritis (RA) angiogenesis remains undefined. In a recent study, we demonstrated that like CCR7 expression, CCL21 expression is elevated on RA synovial tissue lining macrophages and fibroblasts as well as in sublining endothelial cells.

We previously reported that macrophages from RA synovial fluid exhibited higher levels of CCL19 and CCL21 compared with RA and normal peripheral blood cells (6). We also observed that ligation of CCL21 in RA fibroblasts and macrophages induced production of proangiogenic factors such as vascular endothelial growth factor (VEGF), angiogenin 1, and interleukin-8 (IL-8), suggesting that CCL21 plays an indirect role in RA angiogenesis (6). In contrast, other investigations

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<sup>1</sup>Sarah R. Pickens, MA, Nathan D. Chamberlain, BBmE, Shiva Shahrara, PhD: University of Illinois at Chicago; <sup>2</sup>Michael V. Volin, PhD: Midwestern University, Chicago College of Osteopathic Medicine, Downers Grove, Illinois; <sup>3</sup>Richard M. Pope, MD, Nicholas E. Talarico, BS, Arthur M. Mandelin II, MD, PhD: Feinberg School of Medicine, Northwestern University, Chicago, Illinois.

Dr. Mandelin has received speaking fees from Abbott (less than \$10,000) and UCB (more than \$10,000).

Address correspondence to Shiva Shahrara, PhD, University of Illinois at Chicago, Department of Medicine, Division of Rheumatology, E807 MSB, 835 South Wolcott Avenue, Chicago, IL 60612. E-mail: shahrara@uic.edu.

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have shown that CCL19-activated RA synovial tissue fibroblasts produce VEGF, while this effect was not noted with CCL21 stimulation (7). These observations are consistent with the association between CCR7 expression and hypoxia, a process that is essential for the initiation of angiogenesis (8). It was previously shown that hypoxia-inducible factor 1 $\alpha$  (HIF-1 $\alpha$ ) and HIF-2 $\alpha$  are responsible for up-regulating CCR7 levels, and that inhibition of CCR7 and/or the ERK-1/2 signaling pathway significantly suppresses hypoxia-induced cell migration and invasion, thus supporting the role of CCR7 in angiogenesis (8).

In this study, we observed that in the blood vessels of patients with RA, the expression of CCL21 and CCR7 is comparable and demonstrates a linear correlation. Cells in RA synovial tissue lining, including RA fibroblasts and macrophages, activated with CCL21 produce potent proangiogenic factors (6); therefore, the direct role of CCL21 in RA angiogenesis was evaluated. Our results demonstrate that CCL21-induced human microvascular endothelial cell (HMVEC) chemotaxis and tube formation are mediated by CCR7 ligation and activation of the phosphatidylinositol 3-kinase (PI3K) pathway. Furthermore, we demonstrate that CCL21 enhances the formation of blood vessels *in vivo* through recruitment of endothelial cells as well as endothelial progenitor cells (EPCs) in concentrations that are available in RA synovial fluid and tissue. Interestingly, we show that factors in RA synovial fluid can greatly increase endothelial cell CCL21 expression, highlighting the importance of synovial fluid in attracting CCR7-positive cells. Finally, we demonstrate that RA synovial fluid-mediated endothelial cell migration and/or tube formation is significantly reduced by CCL21 and/or CCR7 neutralization. In short, our data suggest that therapy directed against CCR7 ligation in RA may reduce leukocyte migration into the diseased joint by inhibiting angiogenesis.

## MATERIALS AND METHODS

**Antibodies and immunohistochemical analysis.** The studies were approved by the Northwestern University Institutional Review Board, and all donors provided written informed consent. RA synovial tissue samples were obtained from the practices of orthopedic surgeons and were deidentified. RA and normal synovial tissue specimens were fixed in formalin, embedded in paraffin, and sectioned. Synovial tissue specimens were immunoperoxidase-stained using Vector Elite ABC Kits, with diaminobenzidine (DAB) as a chromogen. Slides were deparaffinized in xylene for 20 minutes, followed by rehydration by transfer through graded alcohol. Antigens were unmasked by first incubating the slides in boiling citrate buffer for 15 minutes, followed by type II trypsin digestion for 30 minutes at 37°C. Nonspecific binding of avidin and biotin was blocked using an avidin–biotin block-

ing kit (Vector). Tissue samples were incubated with antibodies to human CCR7 (1:500; R&D Systems), CCL21 (1:67; R&D Systems), lymphatic vessel endothelial hyaluronan receptor 1 (LYVE-1) (1:25; R&D Systems), von Willebrand factor (vWF) (1:1,000; Dako), or an IgG control antibody (Beckman Coulter). For immunohistochemical analysis (Figures 1A, F, and G), the slides were counterstained with Harris' hematoxylin and treated with lithium carbonate for bluing. For studies of the colocalization of CCL21 on vWF-positive cells (Figure 1E), Texas Red-labeled anti-goat antibody (1:200; Abcam) was used to visualize CCL21 staining, and fluorescein isothiocyanate-conjugated anti-rabbit antibody (1:250; Abcam) was used to visualize vWF immunostaining in RA synovial tissues.

Slides were evaluated in a blinded manner by 2 of the authors (AMM and MVV) (9–12). Tissue sections were scored (0–5-point scale) for staining of the lining and endothelium (6,13,14). CCR7- and CCL21-positive blood vessel staining was scored (0–5-point scale) in 12 high-power fields (hpf); blood vessels were identified by morphology in serial sections of CCR7- and CCL21-stained slides. CCR7- and CCL21-positive lymphatic vessel staining was scored in 15 hpf; lymphatic vessels were identified by LYVE-1-positive staining in serial sections of CCR7- and CCL21-stained slides. The correlation of receptor and ligand expression on blood vessels was determined by regression analysis.

To localize CCR7 to macrophages or endothelial cells in RA synovial tissue, slides were deparaffinized, and the antigen was unmasked by proteinase K digestion buffer (Dako) for 10 minutes. An EnVision G2 system (Dako) was used to stain tissue with anti-CCR7 (1:25 dilution; R&D Systems), using DAB (brown staining) as a chromogen. Thereafter, tissues were blocked and stained with anti-vWF (1:1,000; Dako) or anti-CD68 (1:100; Dako), using fast red (red staining) as a chromogen, according to the manufacturer's instructions.

**Cell isolation, culture, and procedures.** Normal peripheral blood mononuclear cells were isolated by Histopaque gradient centrifugation (Sigma-Aldrich). Monocytes were isolated from normal peripheral blood, using a negative selection kit (StemCell Technologies) according to the manufacturer's instructions (6,13). Monocytes were subsequently differentiated to macrophages by culturing in RPMI containing 20% fetal bovine serum for 7 days. Synovial tissue fibroblasts were isolated from fresh RA synovial tissue by mincing and digestion in a solution of Dispase, collagenase, and DNase and used between passages 3 and 9 (15). Macrophages or RA fibroblasts were left untreated or were treated with lipopolysaccharide (LPS) (10 ng/ml; Sigma), tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) (10 ng/ml; R&D Systems), IL-1 $\beta$  (10 ng/ml; R&D Systems), IL-17 (50 ng/ml; R&D Systems), and RA synovial fluid (1:4). Cells were harvested after 6 hours, and CCR7 messenger RNA (mRNA) levels were quantified by real-time reverse transcription–polymerase chain reaction (RT-PCR) (6,13).

Because the number of endothelial cells obtained from RA synovial digestion was very restricted and were therefore easily overgrown by RA synovial tissue fibroblasts, we compared the expression of CCR7 in several primary endothelial cell types in order to select one as a surrogate for RA endothelial cells. Skin and lung HMVECs (Lonza) and human umbilical vein endothelial cells (HUVECs) (Lonza) were cultured in endothelial growth medium EGM-2 MV. In a different experiment, skin HMVECs were either left untreated or were treated with IL-17 (50 ng/ml; R&D Systems) and RA



synovial fluid (1:4) for 6 hours. Subsequently, cells were harvested, and CCR7 or CCL21 mRNA expression was quantified by real-time RT-PCR.

**Quantification of CCR7 or CCL21 expression in different cell types.** RNA was extracted using TRIzol (Invitrogen), and RT and real-time RT-PCR were performed to determine CCR7 and/or CCL21 expression levels, as previously described (16–18). Relative gene expression was determined using the  $\Delta\Delta C_t$  method, and results were expressed as fold increases. To determine levels of CCR7 (1:1,000; R&D Systems) in lung and skin HMVECs as well as HUVECs, Western blot analysis was performed on cell lysates from each cell type, and equal loading was determined by actin blotting (1:3,000; Sigma).

**HMVEC chemotaxis.** To examine chemotaxis, skin HMVECs ( $1.25 \times 10^4$ ) were placed in 48-well Boyden chemotaxis chambers (Neuro Probe) (19,20). The chambers were inverted and incubated at 37°C for 2 hours, allowing cell attachment. The chambers were reinverted, and phosphate buffered saline (PBS), positive control VEGF (10 ng/ml; R&D Systems), CCL19, or CCL21 at concentrations from 0.001 ng/ml to 100 ng/ml (R&D Systems) was added, and chambers were incubated for 2 hours at 37°C. The number of migrating cells was counted, and the data are presented as the average of 3 high-power (40 $\times$ ) fields/well, averaged for each triplicate.

To test the specificity of HMVEC migration induced by CCL21 (1 ng/ml, 10 ng/ml, and 50 ng/ml), HMVECs were blocked with anti-CCR7 antibody or IgG control (10  $\mu$ g/ml) for 1 hour at 37°C (R&D Systems) (18,21). To define which signaling pathway(s) mediated CCL21-induced HMVEC chemotaxis, HMVECs were incubated with inhibitors of PI3K (LY294002; 1  $\mu$ M or 5  $\mu$ M), ERK (PD98059; 1  $\mu$ M or 5  $\mu$ M), JNK (SP600125; 1  $\mu$ M or 5  $\mu$ M), or DMSO for 2 hours in the Boyden chamber with CCL21 (10 ng/ml). To determine the role of CCL21 and/or CCR7 in RA synovial fluid-mediated endothelial cell migration, HMVEC chemotaxis induced by RA synovial fluid was examined following incubation of synovial fluid (1:20) with control IgG or anti-CCL21 antibody (10  $\mu$ g/ml; R&D Systems) (18,21) or treatment of HMVECs with antibodies to CCR7 (10  $\mu$ g/ml) or IgG control (18,21).

**Characterization of CCL21 signaling pathways in HMVECs.** HMVECs were left untreated or were treated with CCL21 (10 ng/ml) for 5–65 minutes. Cell lysates were examined by Western blot analysis, as previously described (16,18,22). The immunoblots were probed with pAkt, pERK, and pJNK (1:1,000; Cell Signaling Technology) and/or with Akt, ERK, and JNK (1:3,000; Cell Signaling Technology) overnight.

**HMVEC tube formation assay.** To perform Matrigel tube formation assays, BD Matrigel Matrix (50  $\mu$ l; BD PharMingen) was polymerized for 30 minutes at 37°C in a 96-well plate. To evaluate which signaling pathways contribute to CCL21-mediated HMVEC tube formation, HMVECs ( $4 \times 10^5$ /ml) were incubated with inhibitors (1  $\mu$ M or 5  $\mu$ M) to PI3K (LY294002), ERK (PD98059), JNK (SP600125), or DMSO for 45 minutes at 37°C prior to adding to polymerized Matrigel. CCL21 (10 ng/ml) was then added to the wells, and the plate was incubated for 16 hours at 37°C. To determine whether CCR7 plays a role in RA synovial fluid-induced HMVEC tube formation, HMVECs were incubated with antibody to CCR7 or IgG for 45 minutes at 37°C. Cells were then added to polymerized Matrigel, and RA synovial fluid (1:20) was added to the wells, followed by incubation for 16 hours at 37°C. Assays for each

condition were performed in triplicate; fibroblast growth factor (FGF; 20 ng/ml) and PBS were used as positive and negative controls, respectively. Thereafter, tube formation was quantified using calcein fluorescent dye (BD PharMingen) according to the manufacturer's instructions. Subsequently, the number of branch points/tubes was quantified as previously described (20,21,23).

**Matrigel plug assay in vivo.** To examine the effect of CCL21 on angiogenesis in vivo, we used a Matrigel plug assay. C57BL/6 mice were injected subcutaneously with 500  $\mu$ l Matrigel containing PBS or basic FGF (bFGF; 100 ng) as negative and positive controls, respectively, and mouse CCL21 (4  $\mu$ g) served as the experimental condition. After 10 days, the mice were killed, and the Matrigel plugs were removed and analyzed for vascularity. For hemoglobin measurement, the plugs were weighed and homogenized, and a serial dilution of methemoglobin was prepared for quantification purposes (21,23). Fifty microliters of supernatant or standard was added to a plate in duplicate, and 50  $\mu$ l of tetramethylbenzidine was added to each sample. The plates were allowed to develop at room temperature, and absorbance was read. To calculate hemoglobin concentrations, the values (gm/dl) were normalized to the weights of the plugs (gm) (21,23). CCL21 levels were also quantified from day 10 Matrigel plug homogenates, using an enzyme-linked immunosorbent assay (R&D Systems).

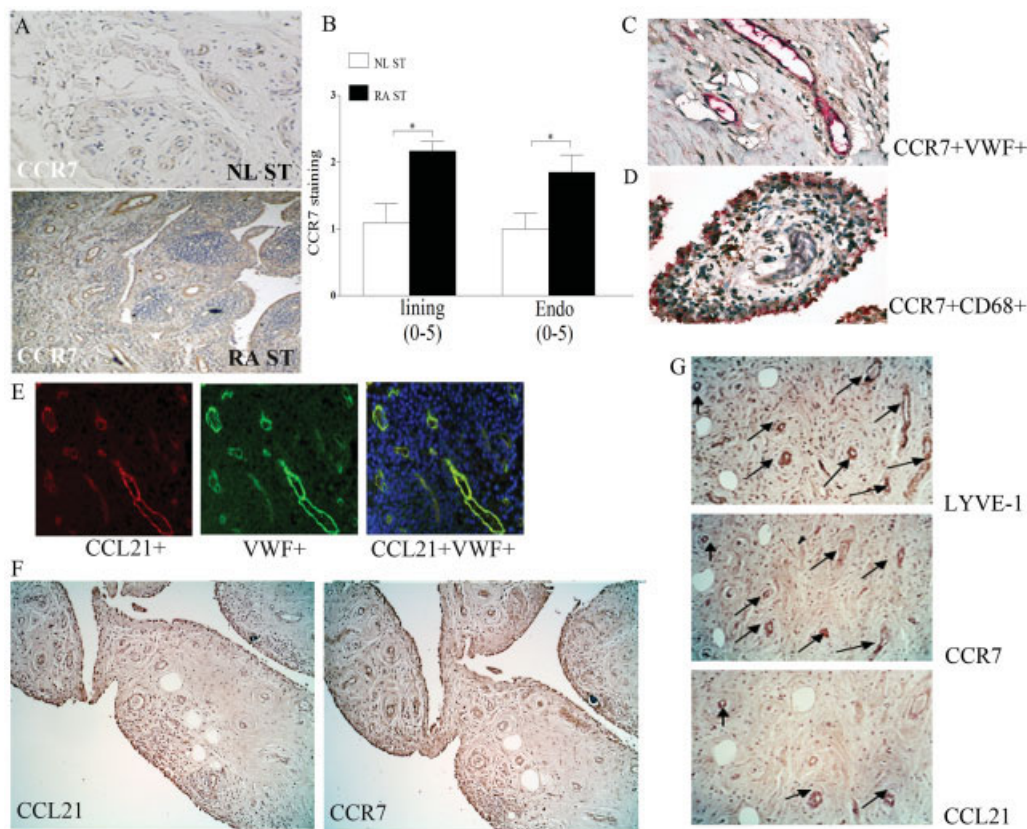
Histology slides from different treatment groups were examined by hematoxylin and eosin (H&E) or Masson's trichrome staining and scored (0–4-point scale) in a blinded manner by 2 observers (AMM and MVV) (14,21). To determine whether Matrigel plugs containing CCL21 were capable of recruiting EPCs, Matrigel plugs from all 3 treatment groups were stained with anti-VEGF receptor 2 antibody (1:25; R&D Systems), which is a known marker for EPC staining. Slides were processed and stained with immunoperoxidase, using Vector Elite ABC Kits with DAB as a chromogen, as described above for the immunohistochemical analysis, with the exception that the antigen did not require unmasking.

**Statistical analysis.** Data were analyzed using Student's 2-tailed *t*-tests for paired and unpaired samples. *P* values less than 0.05 were considered significant.

## RESULTS

**Colocalization of CCR7 and CCL21 in RA synovial tissue sublining endothelial cells.** RA and normal synovial tissue specimens were stained with anti-CCR7 antibody in order to characterize their expression patterns in patients with RA compared with healthy individuals. CCR7 expression was significantly increased in RA synovial tissue lining and endothelial cells compared with normal synovial tissue (Figures 1A and B). We next validated that CCR7 is expressed in RA synovial tissue macrophages in the lining and in endothelial cells in the sublining, by colocalizing CCR7 expression on CD68-positive and vWF-positive cells (Figures 1C and D).

Because the focus of this study was to evaluate angiogenesis in RA mediated by CCL21 ligation to CCR7, studies were performed to colocalize CCL21 and

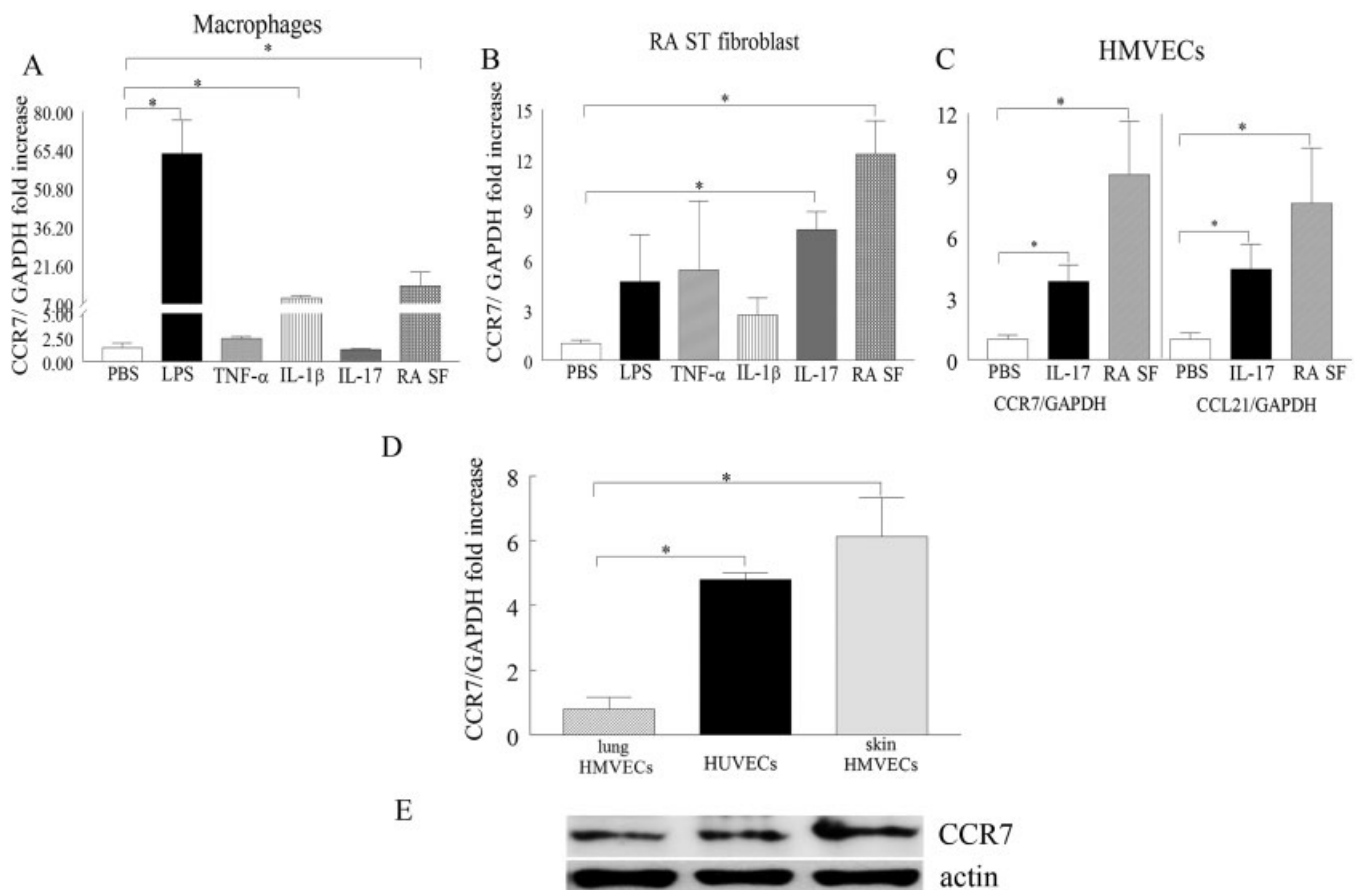


**Figure 1.** CCR7 and CCL21 are colocalized on rheumatoid arthritis (RA) synovial tissue (ST) endothelial (Endo) cells. **A**, Normal (NL) and RA synovial tissue specimens were stained with anti-human CCR7. **B**, Positive immunostaining was scored on a 0–5 scale. Values are the mean  $\pm$  SEM ( $n = 12$ ). \* =  $P < 0.05$ . **C** and **D**, RA synovial tissue sections were stained for CCR7 (brown) and von Willebrand factor (vWF) (red) (**C**) or for CCR7 (brown) and CD68 (red) (**D**) in order to distinguish endothelial cells or macrophages that express CCR7. **E**, Colocalization of CCL21 on vWF-positive cells was evaluated using Texas Red-labeled anti-goat antibody to visualize CCL21 staining (red) and fluorescein isothiocyanate-conjugated anti-rabbit antibody to visualize vWF immunostaining (green) in RA synovial tissue. Cells positive for CCL21 (red) and vWF (green) staining are demonstrated by red and green overlay, which is visualized as yellow staining. **F**, RA synovial tissue serial sections were stained with anti-CCL21 and anti-CCR7 in order to determine colocalization of CCL21 and CCR7 in RA synovium ( $n = 12$ ). **G**, RA synovial tissue serial sections were stained with anti-lymphatic vessel endothelial hyaluronan receptor 1 (anti-LYVE-1), anti-CCR7, or anti-CCL21 to determine whether CCL21 and/or CCR7 was expressed in lymphatic vessels. **Arrows** indicate positive staining for LYVE-1, CCR7, or CCL21. Original magnification  $\times 200$  in **A** and **F**;  $\times 400$  in **C–E** and **G**.

CCR7 expression in vWF-positive cells. Positive staining for CCL21 was observed in the blood vessels of all 12 RA synovial tissue samples examined (Figure 1E), and its expression levels were comparable with and closely correlated with CCR7 immunostaining on blood vessels from the same tissue (Figure 1F) ( $R^2 = 0.41$ ,  $P = 0.02$ ). Given that CCL21 and CCR7 are involved in lymphoid neogenesis, RA synovial tissue serial sections were stained with anti-CCL21, anti-CCR7, or anti-LYVE-1 antibodies to determine whether CCL21 and/or CCR7 was expressed in lymphatic vessels. In RA joints, 8 of the 15 LYVE-1-positive fields did not positively stain for CCL21 (mean  $\pm$  SEM  $0.43 \pm 0.12$ ), while CCR7 was present in all LYVE-1-positive fields ( $1.5 \pm 0.09$ ) (Figure 1G); thus, the CCL21 expression and CCR7 expression on lymphatic vessels were not correlated

( $R^2 = 0.009$ ,  $P = 0.7$ ). These results supported the role of CCL21 and CCR7 in angiogenesis in RA.

**Role of RA synovial fluid in the expression of CCR7 in macrophages and RA fibroblasts and the levels of CCL21 and CCR7 in endothelial cells.** Skin HMVECs may function as a surrogate for RA endothelial cells because of elevated levels of CCR7 on skin compared with lung HMVECs. Because the expression levels of CCR7 and CCL21 were elevated in RA synovial tissue lining as well as sublining endothelial cells, we sought to determine which factors may affect their expression in macrophages, fibroblasts, and endothelial cells. We demonstrated that stimulation with LPS, IL-1 $\beta$ , and RA synovial fluid in macrophages (Figure 2A) and with IL-17 and RA synovial fluid in RA fibroblasts (Figure 2B) significantly increased CCR7 expression



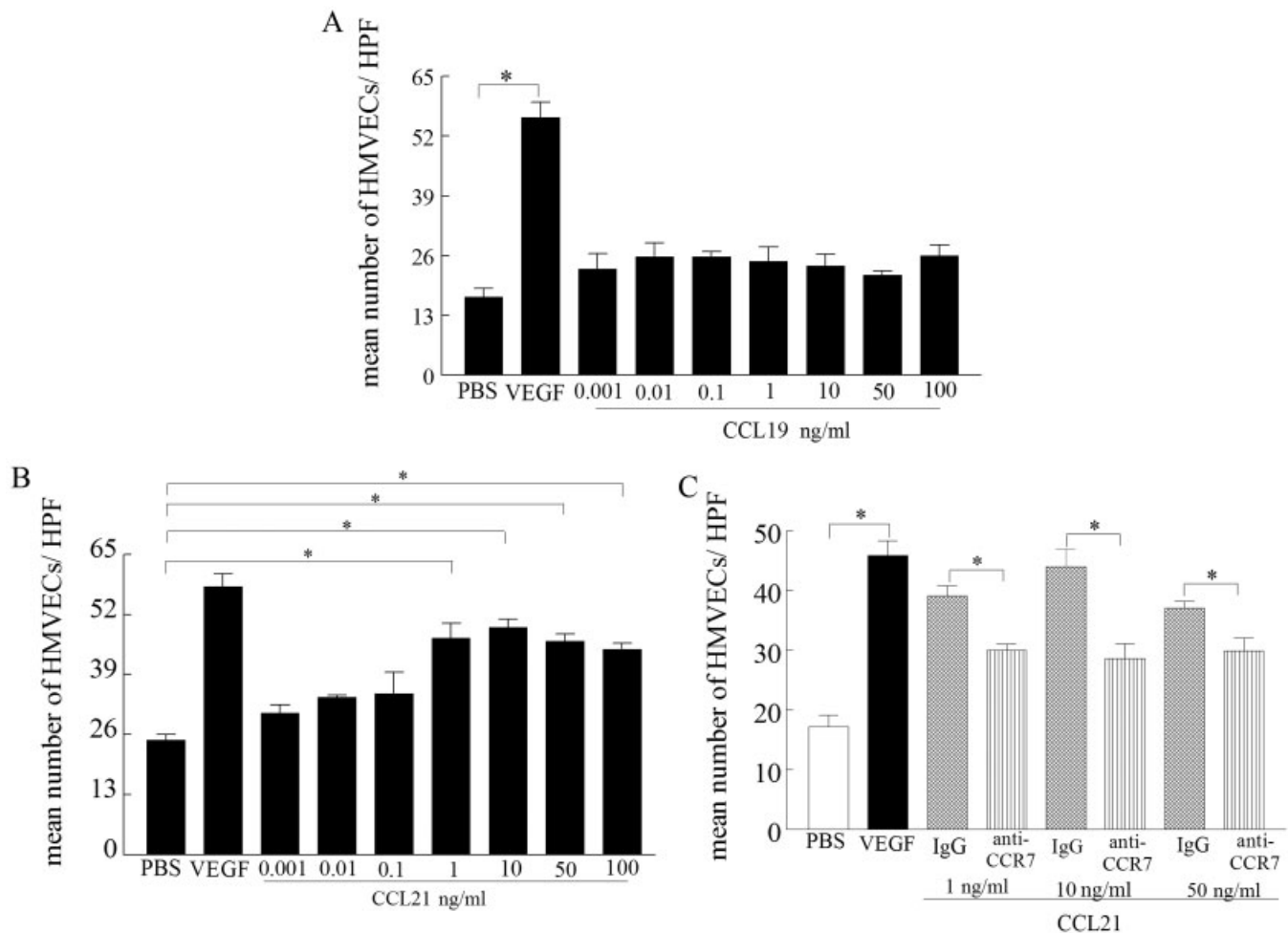
**Figure 2.** Expression of CCR7 and CCL21 is modulated by interleukin-17 (IL-17) or RA synovial fluid (SF) in human microvascular endothelial cells (HMVECs), and skin HMVECs can be used as surrogates for RA endothelial cells because of their elevated levels of CCR7. **A** and **B**, Cells were left untreated (phosphate buffered saline [PBS]) or were treated with lipopolysaccharide (LPS), tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), IL-1 $\beta$ , IL-17, or RA synovial fluid for 6 hours, and expression of CCR7 was determined in peripheral blood in vitro-differentiated macrophages (**A**) or RA fibroblasts (**B**) by real-time reverse transcription-polymerase chain reaction (RT-PCR) ( $n = 5-10$ ). **C**, HMVECs were either left untreated or were treated with IL-17 or RA synovial fluid for 6 hours, and expression of CCR7 and CCL21 was determined by real-time RT-PCR ( $n = 4-5$ ). Bars in **A-C** show the mean  $\pm$  SEM fold increase compared with PBS; the results are normalized to GAPDH. **D**, CCR7 mRNA levels were determined in lung HMVECs, human umbilical vein endothelial cells (HUVECs), and skin HMVECs. Bars show the mean  $\pm$  SEM increase compared with lung HMVECs ( $n = 3-6$ ). **E**, CCR7 protein levels in lung and skin HMVECs and in HUVECs were determined by Western blot analysis; equal loading was determined by actin blotting.  $* = P < 0.05$ . See Figure 1 for other definitions.

levels. Interestingly, in HMVECs, the expression levels of CCR7 and CCL21 were modulated by stimulation with IL-17 and RA synovial fluid (Figure 2C), perhaps explaining the relationship between the expression of this receptor and its ligand on RA blood vessels. Because RA synovial fluid was the common factor capable of driving the expression of CCR7 and/or CCL21 in all of the cell types examined, the role of RA synovial fluid was highlighted in the production of CCL21 and the attraction of CCR7-positive cells. Although RA synovial tissue endothelial cells express CCR7, obtaining sufficient quantities of RA endothelial cells from synovial tissue was not possible, and these cells were not commercially available; therefore, expression of this receptor was compared among endothelial primary cell types.

We observed that the levels of CCR7 expression were comparable in skin HMVECs and HUVECs, and that the expression level in both was greater than that in lung HMVECs (Figures 2D and E). Therefore, skin HMVECs were used as surrogates for RA endothelial cells.

**Induction of endothelial cell (HMVEC) migration by CCL21 but not CCL19.** Next, we performed experiments to determine whether CCR7-corresponding ligands were involved in HMVEC (skin) chemotaxis and/or tube formation. Although CCL21 was chemotactic for HMVECs at concentrations ranging from 1 ng/ml to 100 ng/ml ( $P < 0.05$ ) (Figure 3B), CCL19 did not induce HMVEC migration (Figure 3A). In our previous study, the mean  $\pm$  SEM concentrations of CCL21 in RA synovial fluid samples ( $n = 79$ ) and



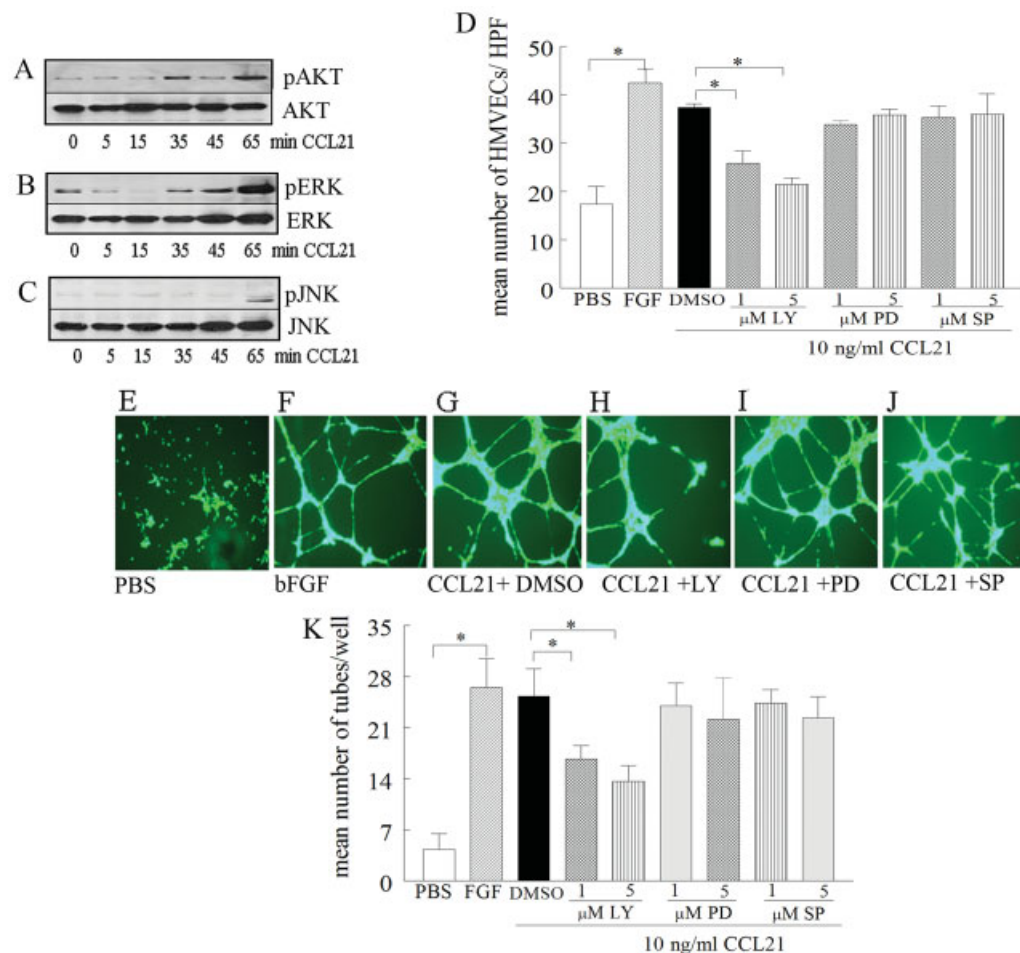


**Figure 3.** CCL21 induces HMVEC migration through binding to CCR7. **A** and **B**, Evaluation of CCL19-induced (**A**) and CCL21-induced (**B**) HMVEC chemotaxis showed that CCL21 induced cell migration but CCL19 did not. **C**, CCL21-induced HMVEC chemotaxis was suppressed by neutralizing antibody to CCR7 but not by IgG control. Bars show the mean  $\pm$  SEM. \* =  $P < 0.05$ . hpf = high-power field; VEGF = vascular endothelial growth factor (see Figure 2 for other definitions).

tissue specimens ( $n = 11$ ) were  $519 \pm 38$  pg/ml (up to 3.4 ng/ml) and  $824 \pm 104$  pg/mg (up to 1.6 ng/mg), respectively (6); these concentrations were highly chemotactic for HMVEC migration. Furthermore, incubation of HMVECs with neutralizing antibody to CCR7 suppressed CCL21-induced HMVEC migration (Figure 3C), suggesting that the chemotactic effect was attributable to CCR7 ligation. These results suggested that CCL21, at concentrations present in the RA joint, can mediate HMVEC migration via CCR7 ligation.

**CCL21 activates the PI3K, ERK, and JNK pathways in HMVECs, but only inhibition of PI3K reduces CCL21-induced HMVEC chemotaxis and tube formation.** To determine which signaling pathways in HMVECs are activated by CCL21, phosphorylation of the MAPK and Akt pathways was evaluated by immunoblot analysis.

Our data demonstrated that CCL21-induced phosphorylation of ERK and Akt-1 began as early as 35 minutes, while JNK was activated later, at 65 minutes (Figures 4A–C). To determine which signaling pathways mediate HMVEC migration, chemical inhibitors at concentrations of 1  $\mu$ M or 5  $\mu$ M were used; a concentration of 10  $\mu$ M was toxic and resulted in cell death, as determined by trypan blue staining (data not shown). Inhibition of ERK and JNK was ineffective in suppressing CCL21-induced HMVEC chemotaxis, while inhibition of PI3K reduced chemotaxis, starting at a concentration of 1  $\mu$ M (Figure 4D). Similarly, although inhibition of PI3K (starting at a concentration of 1  $\mu$ M) decreased CCL21-mediated tube formation by 35–40%, suppression of ERK and JNK had no effect on this process (Figures 4E–K). These results suggested that CCL21-



**Figure 4.** CCL21-induced human microvascular endothelial cell (HMVEC) migration and tube formation are suppressed by phosphatidylinositol 3-kinase (PI3K) inhibition. **A–C**, To determine the mechanism of CCL21 in HMVEC migration, cells were stimulated with CCL21 (10 ng/ml) for 0–65 minutes, and cell lysates were probed for pAkt (**A**), pERK (**B**), or pJNK (**C**) ( $n = 3$ ). **D**, To define which signaling pathways mediated CCL21-induced HMVEC chemotaxis, HMVECs were incubated with inhibitors of PI3K (LY294002 [LY]), ERK (PD98059 [PD]), and JNK (SP600125 [SP]) or with DMSO for 2 hours in a Boyden chamber with CCL21. To examine the mechanism of CCL21-induced tube formation, HMVECs were incubated with antibody to CCR7 or IgG for 45 minutes at 37°C. Cells were then added to polymerized Matrigel, and rheumatoid arthritis synovial fluid was added to the wells, followed by incubation for 16 hours at 37°C. Assays for each condition were performed in triplicate; fibroblast growth factor (FGF) and phosphate buffered saline (PBS) were used as positive and negative controls, respectively. **E–J**, Representative photomicrographs of wells treated with PBS (**E**), basic FGF (bFGF) (**F**), CCL21 plus DMSO (**G**), CCL21 plus LY294002 (**H**), CCL21 plus PD98059 (**I**), and CCL21 plus SP600125 (**J**) are shown. **K**, The numbers of tubes per well were determined. Bars in **D** and **K** show the mean  $\pm$  SEM. \* =  $P < 0.05$ . hpf = high-power field. Original magnification  $\times 400$  in **E–J**. Color figure can be viewed in the online issue, which is available at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

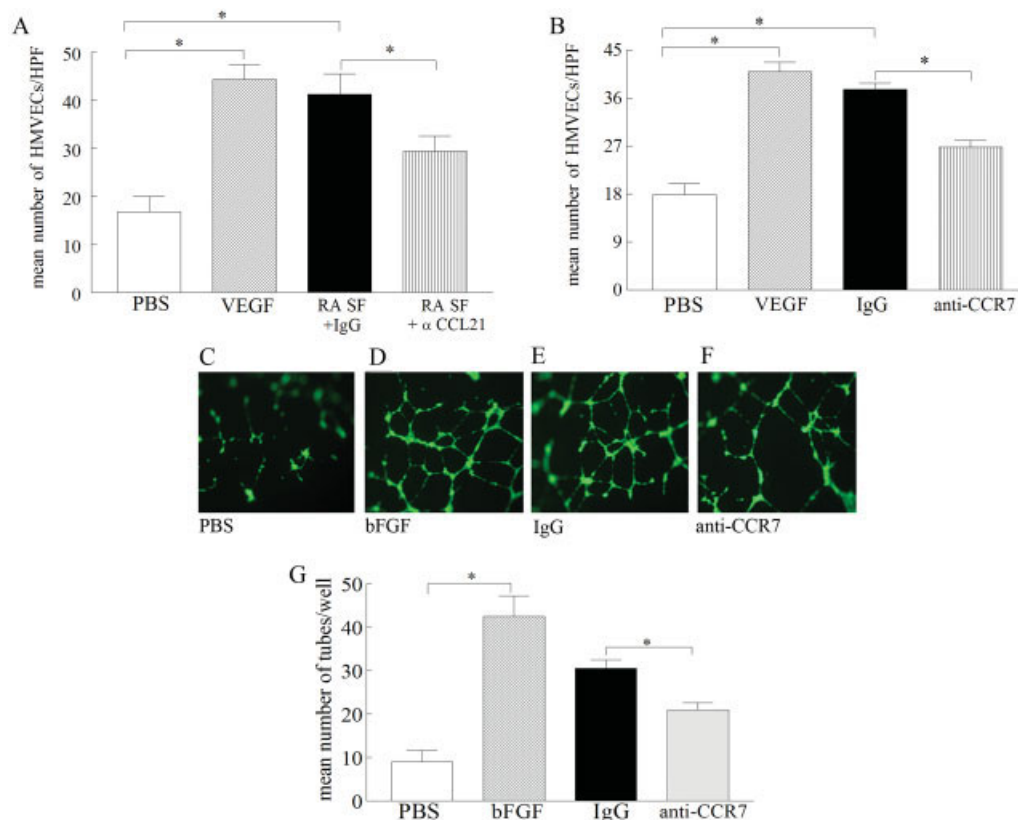
induced HMVEC chemotaxis and tube formation are mediated through the PI3K/Akt-1 pathway.

**Involvement of CCL21 and CCR7 in RA synovial fluid-mediated HMVEC chemotaxis.** Next, we sought to determine whether the CCL21 identified in human RA synovial fluid is chemotactic for HMVECs. We observed that RA synovial fluid immunodepleted by anti-CCL21 significantly reduced HMVEC chemotaxis compared with control IgG-treated fluid (Figure 5A). Neutralization of CCR7 on HMVECs was effective in suppressing

RA synovial fluid-mediated HMVEC migration (Figure 5B) and tube formation (Figures 5C–G) by 30%. These results suggested that CCL21 and CCR7 may play an important role in angiogenesis in RA.

**CCL21-induced angiogenesis in vivo in Matrigel plugs.** The role of CCL21 in angiogenesis in vivo was assessed by determining its effect on blood vessel formation in Matrigel plugs by quantifying hemoglobin levels or histologic staining. The hemoglobin content in the CCL21-treated group was 14-fold greater ( $P < 0.05$ )





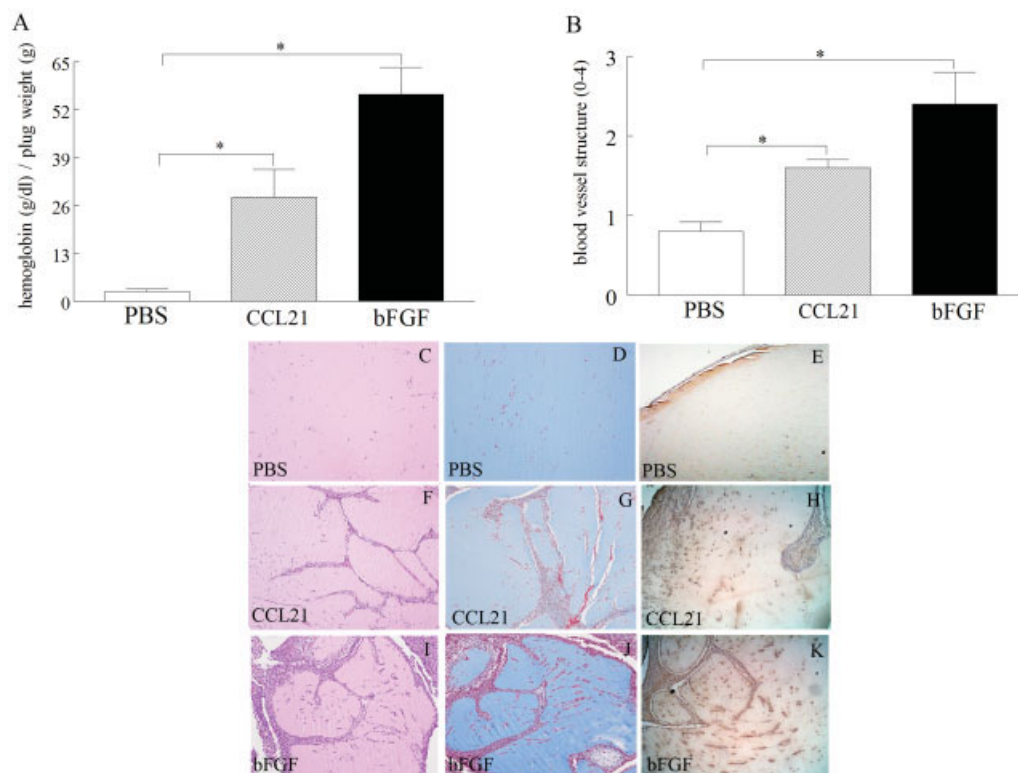
**Figure 5.** Rheumatoid arthritis (RA) synovial fluid (SF)-induced HMVEC chemotaxis and/or tube formation is mediated by CCL21 ligation to CCR7. **A**, Anti-CCL21 or control IgG was added to RA synovial fluid specimens ( $n = 8$ ) for 1 hour at  $37^{\circ}\text{C}$  prior to evaluating HMVEC chemotaxis in response to RA synovial fluid. **B**, HMVECs were incubated with antibodies to CCR7 as well as isotype control for 1 hour prior to evaluating HMVEC chemotaxis in response to synovial fluid. Cells were then added to polymerized Matrigel, and RA synovial fluid was added to the wells, followed by incubation for 16 hours at  $37^{\circ}\text{C}$ . **C–F**, Representative photomicrographs of wells treated with PBS (**C**), bFGF (**D**), IgG plus RA synovial fluid (**E**), and anti-CCR7 plus RA synovial fluid (**F**) are shown. **G**, The mean numbers of tubes per well were determined. Bars in **A**, **B**, and **G** show the mean  $\pm$  SEM of 9 synovial fluid samples examined in 3 independent tube formation studies.  $* = P < 0.05$ . See Figure 4 for other definitions. Original magnification  $\times 400$  in **C–F**. Color figure can be viewed in the online issue, which is available at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

than that in the PBS control group (Figure 6A). Blood vessel formation in Matrigel plugs was also examined histologically, using H&E (Figures 6C, F, and I) and Masson's trichrome staining (Figures 6D, G, and J). The histologic analysis demonstrated that CCL21 markedly enhanced blood vessel growth compared with control ( $P < 0.05$ ) (Figure 6B). The mean  $\pm$  SEM concentration of CCL21 quantified from day 10 Matrigel plugs was  $405 \pm 89$  pg/ml, which is within the range of CCL21 levels detected in RA synovial fluid ( $519 \pm 38$  pg/ml) and tissue ( $824 \pm 104$  pg/mg). We also evaluated whether Matrigel plugs containing CCL21 were capable of recruiting EPCs. We documented that CCL21-induced EPC extravasation was significantly greater than that in the control group, suggesting that both endothelial cells and EPCs contribute to CCL21-induced in vivo blood vessel formation (Figures 6E, H, and K). These

results support the role of CCL21 in angiogenesis in vivo at concentrations available in RA synovial fluid and tissue.

## DISCUSSION

Our group previously demonstrated that CCR7 and CCL21 are coexpressed on synovial tissue endothelial cells, and that the CCR7 ligands CCL19 and CCL21 are capable of inducing macrophages and RA fibroblasts to produce potent proangiogenic factors (6). Therefore, we undertook studies to determine whether CCL19 or CCL21 ligation to CCR7 may directly contribute to angiogenesis in RA. Our data demonstrated that although CCL21 induces HMVEC chemotaxis, CCL19 was not effective in this process. We further demonstrated that CCL21-mediated HMVEC chemotaxis and/or tube formation is mediated through ligation to



**Figure 6.** CCL21 enhances blood vessel growth in Matrigel plugs in vivo. The role of CCL21 in angiogenesis in vivo was assessed by determining its effect on blood vessel formation in Matrigel plugs by quantifying hemoglobin levels and histologic staining. **A**, The hemoglobin content in the CCL21-treated group was 14-fold greater than that in the phosphate buffered saline (PBS) control group. Matrigel containing basic fibroblast growth factor (bFGF) served as a positive control. Bars show the mean  $\pm$  SEM ( $n = 10$ ). **B**, Blood vessel growth was quantified on a 0–4-point scale in Matrigel plugs containing PBS, CCL21, or bFGF. CCL21 markedly enhanced blood vessel growth compared with control. Bars show the mean  $\pm$  SEM ( $n = 5$ ). \* =  $P < 0.05$ . **C–K**, Blood vessel formation in Matrigel plugs was examined histologically, using hematoxylin and eosin (**C**, **F**, and **I**) and Masson's trichrome staining (**D**, **G**, and **J**) of blood vessels in paraffin sections of Matrigel plugs containing PBS (**C–E**), CCL21 (**F–H**), and bFGF (**I–K**). Staining with vascular endothelial growth factor receptor 2 (**E**, **H**, and **K**) was performed to distinguish epithelial progenitor cell recruitment. Original magnification  $\times 200$ . Color figure can be viewed in the online issue, which is available at [http://onlinelibrary.wiley.com/journal/10.1002/\(ISSN\)1529-0131](http://onlinelibrary.wiley.com/journal/10.1002/(ISSN)1529-0131).

CCR7 on HMVECs and activation of the PI3K pathway. Last, we documented that CCL21, at concentrations detected in the RA joint, can induce the formation of blood vessels. Therefore, these results support a novel role for CCL21 in angiogenesis in RA.

CCL21 and CCR7 colocalized on RA endothelium, where their expression was correlated. Conversely, results from previous studies showed that CCL21 was only weakly expressed on RA blood vessels, while demonstrating that 100% of CCL21-producing vessels were LYVE-1-positive in RA synovium (24). Our data demonstrate that close to 50% of LYVE-1-positive fields positively stained for CCL21, while CCR7 immunostaining was present on all LYVE-1-positive fields. The variability of these results may be attributable in part to the heterogeneity of RA disease or patient treatment. Hence, the RA synovium used in studies by Manzo et al (24) may have been obtained from patients

with lower disease activity, because only 12 of 27 patients were receiving substantial treatment, and, as a result, inflammatory factors such as IL-17 that elevate the expression of CCL21 may not have been present or may have been expressed at lower concentrations. Thus, the intensity of CCL21 immunostaining on blood vessels was weak. Unlike the findings reported by Manzo et al (24), the lower percentage of CCL21 staining that we observed in LYVE-1-positive cells may be attributable to the selection of RA synovial tissue. Although Manzo and colleagues selected 9 RA tissue specimens that were rich in lymphoid aggregates, we performed CCL21 and LYVE-1 immunostaining using randomly selected RA synovium.

We previously demonstrated that in macrophages, stimulation with RA synovial fluid modulated the expression of CCR7 and CCL21 (6). Furthermore, activation with IL-17 and RA synovial fluid significantly in-

creased the expression levels of CCR7 in RA fibroblasts and endothelium as well as the concentrations of CCL21 in endothelial cells. Although in HMVECs, TNF $\alpha$  treatment had no effect on CCL21 expression (data not shown), levels of this chemokine were greatly elevated in human dermal lymphatic endothelial cells stimulated with TNF $\alpha$  (25), suggesting that expression of CCL21 is differentially regulated in lymphatic vessels compared with blood vessels. HIF-1 $\alpha$  is a hypoxic transcription factor that has been shown to modulate CCR7 expression (8) and, similar to its downstream target, is expressed in RA synovial tissue macrophages and endothelial cells (26). Like CCR7, HIF-1 $\alpha$  expression levels are modulated by Toll-like receptor 4 (TLR-4) ligation in RA synovial fluid macrophages (26), suggesting that TLR-4 endogenous ligands in RA synovial fluid (27) may be important for regulation of the HIF-1 $\alpha$  and CCR7 pathways.

Consistent with our findings in RA synovial tissue, skin HMVECs expressed elevated levels of CCR7 compared with controls; therefore, these cells were used as surrogates for RA endothelial cells to examine the direct effect of CCL19 and CCL21 on angiogenesis. We demonstrated that CCL21 induces HMVEC chemotaxis at concentrations available in the human RA joint, which is due to its ligation to CCR7. Unlike CCL21, CCL19 had no effect on HMVEC migration. Although CCL19 and CCL21 have similar affinity to CCR7, ligation of these chemokines mediates different signaling effects. CCL19, but not CCL21, activates CCR7 phosphorylation and internalization, resulting in receptor desensitization (28,29). This suggests that CCR7-induced cell responses to CCL19 may be associated with a shorter time span compared with those of CCL21. Consistently, previous studies have shown that although CCL19 was chemotactic for RA synovial tissue fibroblasts, CCL21 was unable to attract these cells (7). Although CCL19-activated RA synovial tissue fibroblasts produce VEGF, this effect was not observed with CCL21 stimulation (7). These results suggest that CCL19 or CCL21 ligation of CCR7 can differentially modulate angiogenesis through induction of different signaling pathways.

Next, experiments were performed to investigate signaling pathways that were associated with CCL21-induced HMVEC chemotaxis and tube formation. Inhibition of the CCL21-activated pathways in HMVECs demonstrated that only activation of PI3K significantly reduced CCL21-mediated chemotaxis and tube formation, and suppression of the ERK and JNK pathways had no effect on this process. Consistently, B cell chemotaxis mediated by CCL21 was markedly reduced by PI3K inhibitors, while suppression of the ERK and JNK pathways was ineffective (30). In contrast, monocyte-

derived dendritic cell migration in response to CCL21 was dependent on phospholipase C but not on the PI3K signaling pathway (31). Furthermore, other investigators have shown that PI3K signaling plays an important role in VEGF- and FGF-mediated endothelial cell migration (32–34), suggesting that PI3K is involved in the mediation of angiogenesis by various inflammatory factors.

Because both CCL19 expression and CCL21 expression are highly elevated in RA synovial fluid (6), the contribution of these 2 chemokines was examined in RA synovial fluid-mediated HMVEC chemotaxis. Neutralization of CCL21 but not CCL19 (data not shown) in RA synovial fluid partially reduced RA synovial fluid-mediated HMVEC chemotaxis. RA synovial fluid-mediated HMVEC chemotaxis was also mediated through CCR7, confirming the importance of this receptor in CCL21-mediated angiogenesis. Similar to CCL21 (6), other pro-angiogenic factors present in human RA synovial fluid are mostly produced by RA synovial tissue fibroblasts (VEGF, bFGF, vascular cell adhesion molecule 1, IL-6, and ELR-positive CXC chemokines) or macrophages (TNF $\alpha$ , IL-8, and IL-1 $\beta$ ) (35). Previous studies demonstrated that although both CCL19 and CCL21 play a key role in the migration of T cells (36), neutrophils (37), and dendritic cells (25,38), expression of CCL21 in CCL19<sup>-/-</sup> mice was sufficient for dendritic cell trafficking, maturation, and function, suggesting that CCL21 may be the more critical CCR7 ligand in the inflammatory process (38).

We next demonstrated that CCL21, at concentrations detected in RA synovial fluid and tissue, can directly contribute to blood vessel formation through extravasation of endothelial cells as well as EPCs, because the Matrigel plugs are acellular, and other pro-angiogenic factors cannot be produced.

In conclusion, endothelial cell migration and tube formation induced by CCL21 were mediated through activation of the PI3K pathway and ligation to CCR7. Neutralization of CCL21 or CCR7 significantly down-regulated RA synovial fluid-mediated endothelial cell migration, suggesting that CCL21 plays an important role in RA angiogenesis.

## AUTHOR CONTRIBUTIONS

All authors were involved in drafting the article or revising it critically for important intellectual content, and all authors approved the final version to be published. Dr. Shahrara had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

**Study conception and design.** Pickens, Shahrara.

**Acquisition of data.** Pickens, Chamberlain, Volin, Talarico, Mandelin, Shahrara.

**Analysis and interpretation of data.** Pickens, Chamberlain, Volin, Pope, Shahrara.



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EXTENDED REPORT

# Characterising the expression and function of CCL28 and its corresponding receptor, CCR10, in RA pathogenesis

Zhenlong Chen,<sup>1</sup> Seung-Jae Kim,<sup>1</sup> Abdul B Essani,<sup>1</sup> Michael V Volin,<sup>2</sup> Olga M Vila,<sup>1</sup> William Swedler,<sup>1</sup> Shiva Arami,<sup>1</sup> Suncica Volkov,<sup>1</sup> Latrrese V Sardin,<sup>1</sup> Nadera Sweiss,<sup>1</sup> Shiva Shahrara<sup>1</sup>

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<sup>1</sup>Division of Rheumatology, Department of Medicine, University of Illinois, Chicago, Illinois, USA

<sup>2</sup>Department of Microbiology & Immunology, Midwestern University, Chicago College of Osteopathic Medicine, Downers Grove, Illinois, USA

## Correspondence to

Dr Shiva Shahrara, University of Illinois at Chicago, Department of Medicine, Division of Rheumatology, MSB 835 S Wolcott Ave, E807-E809, Chicago, IL 60612, USA; [shahrara@uic.edu](mailto:shahrara@uic.edu)

ZC and S-JK contributed equally.

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## ABSTRACT

**Objective** This study was conducted to determine the expression pattern, regulation and function of CCL28 and CCR10 in rheumatoid arthritis (RA) pathogenesis.

**Methods** Expression of CCL28 and CCR10 was assessed in RA compared with other arthritis synovial tissues (STs) or fluids (SFs) by histology or ELISA. The factors modulating CCL28 and CCR10 expression were identified in RA myeloid and endothelial cells by ELISA, FACS and Western blotting. The mechanism by which CCL28 ligation promotes RA angiogenesis was examined in control and CCR10-knockdown endothelial cell chemotaxis and capillary formation.

**Results** CCL28 and/or CCR10 expression levels were accentuated in STs and SFs of patients with joint disease compared with normal controls and they were predominately coexpressed in RA myeloid and endothelial cells. We show that protein expression of CCL28 and CCR10 was modulated by tumour necrosis factor (TNF)- $\alpha$  and toll-like receptor 4 ligation in RA monocytes and endothelial cells and by interleukin (IL)-6 stimulation in RA macrophages. Neutralisation of CCL28 in RA SF or blockade of CCR10 on human endothelial progenitor cells (EPCs) significantly reduced SF-induced endothelial migration and capillary formation, demonstrating that ligation of joint CCL28 to endothelial CCR10+ cells is involved in RA angiogenesis. We discovered that angiogenesis driven by ligation of CCL28 to CCR10 is linked to the extracellular signal regulated kinase (ERK) cascade, as CCR10-knockdown cells exhibit dysfunctional CCL28-induced ERK signalling, chemotaxis and capillary formation.

**Conclusions** The overexpression of CCL28 and CCR10 in RA ST and their contribution to EPC migration into RA joints support the CCL28/CCR10 cascade as a potential therapeutic target for RA.

Mucosa-associated epithelial chemokine or CCL28 is a CCR10 ligand, which is typically secreted from epithelial cells in the gut, lung, breast and salivary gland.<sup>1 2</sup> CCL28 is also constitutively expressed in epithelial colon cells where its expression level is modulated by proinflammatory cytokines and bacterial products, indicating that it may play a role in attracting CCR10+ cells to the site of colonic inflammation.<sup>3 4</sup>

Previous studies have shown that CCL28 can recruit CCR10+ IgA or IgE antibody-secreting cells to intestinal and non-intestinal mucosal tissues

or respiratory epithelium in asthma.<sup>5–7</sup> With the use of CCR10-deficient mice, it has been documented that, while localisation and accumulation of IgA antibody-producing plasma cells were compromised in lactating mammary glands, the presence of these cells were minimally affected in gastrointestinal tract.<sup>8</sup> However, despite similar numbers of IgA-antibody producing plasma cells in wild-type and CCR10<sup>−/−</sup> mice, intestinal IgA response and memory B-cell maintenance were severely dysregulated in the knockout mice, highlighting the importance of CCR10 in IgA antibody-producing plasma-cell and B-cell function.<sup>9</sup>

Others have shown that trafficking of CCR10-expressing T regulatory cells (Tregs) detected around bile ducts is facilitated by CCL28 secreted from biliary epithelial cells.<sup>10</sup> A recent elegant study has also revealed that, in ovarian cancer cells, CCL28 modulated by hypoxia facilitates homing of Tregs, which foster ovarian cancer tumour angiogenesis by increasing vascular endothelial growth factor (VEGF) production.<sup>11</sup> These two studies identify dual roles for CCL28-induced Treg extravasation. The study conducted by Eksteen and colleagues<sup>10</sup> identifies an anti-inflammatory role for CCL28-recruited Tregs in the mucosal sites, and, in contrast, the latter study reports that the Tregs attracted to CCL28 produced by ovarian cancer cells sustain tumour growth by enhancing tolerance and angiogenesis.<sup>11</sup>

Rheumatoid arthritis (RA) is a chronic autoimmune disease in which development of new blood vessels facilitates ingress of leucocytes and pannus formation leading to joint deformity and severe disability.<sup>12</sup> Angiogenesis is an early and critical event in RA pathogenesis, which is fostered by an imbalance of joint proinflammatory cytokines and chemokines.<sup>13 14</sup> Earlier studies have shown that ligation of CCL28 to CCR10 is involved in B- and T-cell trafficking,<sup>15</sup> yet the expression pattern, modulating factors and mechanism by which this cascade mediates its pathogenic effect are completely unknown in RA.

We show for the first time that expression of CCL28 and CCR10 is markedly higher in RA and osteoarthritis (OA) synovial tissue (ST) lining macrophages and sublining endothelial cells than in normal (NL) ST. We found that CCL28 and CCR10 expression is modulated by overlapping proinflammatory factors in RA myeloid and

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## Basic and translational research

endothelial cells, where this ligand and receptor pair colocalise. We reveal that CCL28 strongly attracts endothelial cells at the physiological concentration available in RA synovial fluid (SF). Last, we document that knockdown of endothelial CCR10 significantly reduces CCL28-mediated endothelial migration and capillary formation through an extracellular signal-regulated kinase (ERK)-dependent mechanism.

### MATERIALS AND METHODS

#### Antibodies and immunohistochemical analysis

The studies were approved by the institutional review board, and all donors gave informed written consent. STs were deidentified; therefore disease severity and treatment information is unavailable. RA, OA and NL STs were formalin-fixed, paraffin-embedded and sectioned in the pathology core facility. STs were immunoperoxidase-stained using Vector Elite ABC kits (Vector Laboratories, Burlingame, California, USA), with diaminobenzidine (Vector Laboratories) as a chromogen. Briefly, slides were deparaffinised in xylene and then rehydrated by transfer through graded alcohols. Antigens were unmasked by incubating slides in proteinase K digestion buffer (DakoCytomation, Carpinteria, California, USA). Tissues were incubated with antibodies to human CCR10 (1:50; Santa Cruz Biotechnology, Santa Cruz, California, USA) or human CCL28 (1:50; Santa Cruz Biotechnology) or an IgG control antibody. Tissue sections were scored for lining, sublining macrophages and endothelial cell staining on a 0–5 scale by blinded observers (SJK and MVV).<sup>16–20</sup> Scored data were pooled, and the mean±SEM was calculated in each data group. To demonstrate colocalisation of CCL28 and CCR10 on macrophages and endothelial cells, RA ST serial sections were stained with antibodies to CCL28, CCR10, von Willebrand factor (VWF; 1:1000; Vector Laboratories) and CD68 (1:100; Vector Laboratories). To demonstrate cell purity, endothelial progenitor cells (EPCs) isolated from NL blood were stained for classical endothelial markers, VWF (1:1000), VE-cadherin (1:200) or IgG control, and positive staining was overlapped on 4',6-diamidino-2-phenylindole (DAPI)-positive cells (1:1000). Fluorescent images were obtained using a Zeiss LSM 510 confocal microscope.

#### Cell isolation, culture and treatment

NL and RA peripheral blood (PB) and RA SF mononuclear cells were isolated by Ficoll–Paque gradient centrifugation as described previously.<sup>18–22</sup> Monocytes were isolated from NL and RA PB using a negative selection kit, according to the manufacturer's instructions.<sup>18–22</sup> Monocytes were subsequently differentiated to macrophages by culturing in 20% fetal bovine serum (FBS)-containing medium.<sup>18–22</sup> EPCs were isolated from NL blood. Briefly, NL PB mononuclear cells (PBMCs) were isolated and cultured on a collagen-coated plate in 10% FBS-containing endothelial basal medium-2 (EBM-2) medium. After 1 day, non-adherent cells were discarded, and medium was replaced daily for 2–4 weeks. Subsequently, endothelial colonies were isolated by a colony ring and transferred to a new plate where only a homogeneous population of endothelial cells was growing.<sup>23</sup>

RA PB monocytes, RA PB in vitro differentiated macrophages, and human umbilical vein endothelial cells (HUVECs; Lonza, Walkersville, Maryland, USA) were either untreated or treated with 10 ng/mL lipopolysaccharide (LPS; Invivogen, San Diego, California, USA), tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$  or IL-6 or 50 ng/mL IL-17 (R&D Systems, Minneapolis, Minnesota, USA) for 24–72 h before detection of CCL28 production by ELISA or CCR10 expression by FACS analysis

(1:1000; Biolegend) or Western blotting (1:1000; Santa Cruz Biotechnology).

#### Reverse transcriptase (RT)-quantitative PCR

Total cellular RNA was extracted from the different cell types using TRIzol. Subsequently, reverse transcription and real-time RT-PCR were performed to determine CCL28 and CCR10 expression levels as described previously.<sup>18–22</sup> Relative gene expression was determined by the  $\Delta\Delta C_t$  method based on glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels, and results are expressed as fold increase over conditions indicated in the figure legends. CCL28 and CCR10 mRNA expression was quantified in RA PB monocytes treated with disease-modifying antirheumatic drugs (DMARDs) or anti-TNF- $\alpha$  with or without DMARDs.

#### RA patient population

RA specimens were obtained from patients with RA, diagnosed according to the 1987 revised criteria of the American College of Rheumatology.<sup>25</sup> PB was obtained from 30 patients (27 women and 3 men; mean±SD age 51±20.2 years). At the time of evaluation, patients were receiving treatment with non-biological DMARDs (methotrexate, leflunomide, Plaquenil, sulfasalazine, imuran, Minocin or prednisone) alone (n=15; 13 women and 2 men; mean age 46.4±20.0 years), DMARDs plus TNF- $\alpha$  (Humira, Enbrel, Remicade or Cimzia) (n=13; 12 women and 1 man; mean age 55.6±16.4 years) or TNF- $\alpha$  alone (n=2; both women; mean age 61.5 years). These studies were approved by the University of Illinois at Chicago Institutional Ethics Review Board, and all donors gave informed written consent. The total number of patients was 30, but the reader is referred to the figure legends for the exact number of patients in each experiment.

#### Quantification of human CCL28

A human CCL28 ELISA kit (R&D Systems) was used according to the manufacturer's instructions to quantify concentrations of CCL28 in STs (RA, OA and NL), SFs (RA, gout, OA and psoriatic arthritis (PsA)), sera (RA, OA and NL) and RA myeloid and endothelial cells treated with inflammatory factors.

#### Endothelial cell chemotaxis or tube formation

HUVECs were placed in a 48-well Boyden chemotaxis chamber to examine endothelial migration in response to various concentrations of CCL28 (0.001–100 ng/mL; R&D Systems), phosphate-buffered saline (PBS) (negative control) and VEGF (positive control; 10 ng/mL; R&D Systems).<sup>17</sup> The number of migrating cells was counted, and the data represent an average of three high-power fields (HPFs)×40/well, averaged for each triplicate normalised to random migration in the PBS group.

To determine the role of CCL28 and/or CCR10 in RA SF-mediated endothelial migration, HUVEC chemotaxis or tube formation induced by RA SFs was examined after incubation of SFs (1:20) with control IgG or antibody to CCL28 (10  $\mu$ g/mL) or treatment of HUVECs with antibodies to CCR10 (10  $\mu$ g/mL; R&D Systems) or IgG control. The total number of branch points/tubes was quantified per well, and the data represent the mean of three wells normalised to the number of tubes in the PBS control well. Next, the contribution of RA SF CCL28 was confirmed in endothelial chemotaxis using EPCs isolated from human blood that expressed classical endothelial markers (VWF and VE-cadherin) and could form tubes in response to VEGF.

To examine which signalling pathway(s) contribute to CCL28-mediated endothelial chemotaxis and tube formation,

HUVECs were incubated with inhibitors (5  $\mu$ M) to ERK (U0126; with and without CCL28 addition), p38 (SB203580), phosphatidylinositol 3-kinase (PI3K) (LY294002), Jun N-terminal kinase (JNK) (SP600125) or dimethyl sulfoxide (DMSO) for 45 min before addition of CCL28 (50 ng/mL) to the wells.<sup>17</sup>

To confirm that CCL28 ligation to CCR10 is interconnected to activation of the ERK pathway and endothelial function, control and CCR10-knockdown cells were examined for endothelial CCR10 reduction (1:1000) and CCL28-induced ERK activation (CCL28; 100 ng/mL) by Western blotting. Control and CCR10-knockdown cells were also assessed for endothelial chemotaxis and capillary formation in response to CCL28 (50 ng/mL).

### CCR10 silencing in endothelial cells

HUVECs were transfected with CCR10-specific and non-specific control small interfering RNA (Santa Cruz Biotechnologies) at a final concentration of 100 nM using TransIT (Mirus, Madison, Wisconsin, USA) transfection reagent, complying with the manufacturer's instruction with a few modifications. The transfected cell culture medium was replaced after 24 h, and the endothelial cells were used after 72 h of transfection.

### CCL28-activated signalling pathway(s) in the endothelial cells

HUVECs were either untreated or treated with CCL28 (10 or 100 ng/mL) for 0–60 min. Cell lysates were examined by Western blot analysis, and blots were probed with phospho(p) ERK, p-p38 mitogen-activated protein kinase (MAPK), p-protein kinase B (AKT)1 or pJNK (1:1000) overnight and then reprobed with ERK, p38, AKT, JNK or actin (1:3000). CCR10-knockdown and control cells untreated or stimulated by CCL28 (100 ng/mL) were probed for CCR10 and pERK (1:1000) as well as ERK and actin (1:3000). Cell lysates from EPCs or HUVECs were probed for CCR10 or actin expression. Densitometric analysis of the Western blot bands was performed using Image J software.

### Statistical analysis

One-way analysis of variance was used for comparisons among multiple groups, followed by Student's post hoc two-tailed t test. Student's paired and unpaired two-tailed tests were used for comparisons between two groups.  $p < 0.05$  was considered significant.

## RESULTS

### Colocalisation of CCL28 and CCR10 in RA ST myeloid and endothelial cells

When expression of CCL28 and CCR10 was examined in RA compared with OA and NL ST, we found that individuals with RA and OA have markedly higher levels of CCL28 and CCR10 expression on ST macrophages and endothelial cells (figures 1A, B, 2A,B and online supplementary figure S1). Next, colocalisation of CCL28 and CCR10 on RA ST myeloid cells and blood vessels was validated by serial section staining of CCL28 and CCR10 to CD68- and VWF-positive cells (figure 2C,D). Consistent with our histological studies, we document that patients with arthropathies express elevated levels of CCL28 in their ST (RA and OA) and SF (RA, OA, gout, PsA) compared with NL individuals (assessed using ST or serum) (figure 1C,D). These results suggest that, in patients with joint-related disease, the inflammatory response may contribute to production of

CCL28 from myeloid and endothelial cells relative to normal controls. Interestingly, we found that transcription levels of both CCL28 and CCR10 are increased in RA compared with NL myeloid cells. While RA macrophages differentiated from monocytes exhibit a greater trend for CCL28 expression, CCR10 expression trend is higher in RA monocytes than differentiated macrophages (figures 1E and 2E). On the basis of these results, the effect of RA inflammatory mediators on myeloid and endothelial CCL28 and CCR10 expression was evaluated.

### CCL28 and CCR10 are similarly regulated in RA monocytes and endothelial cells

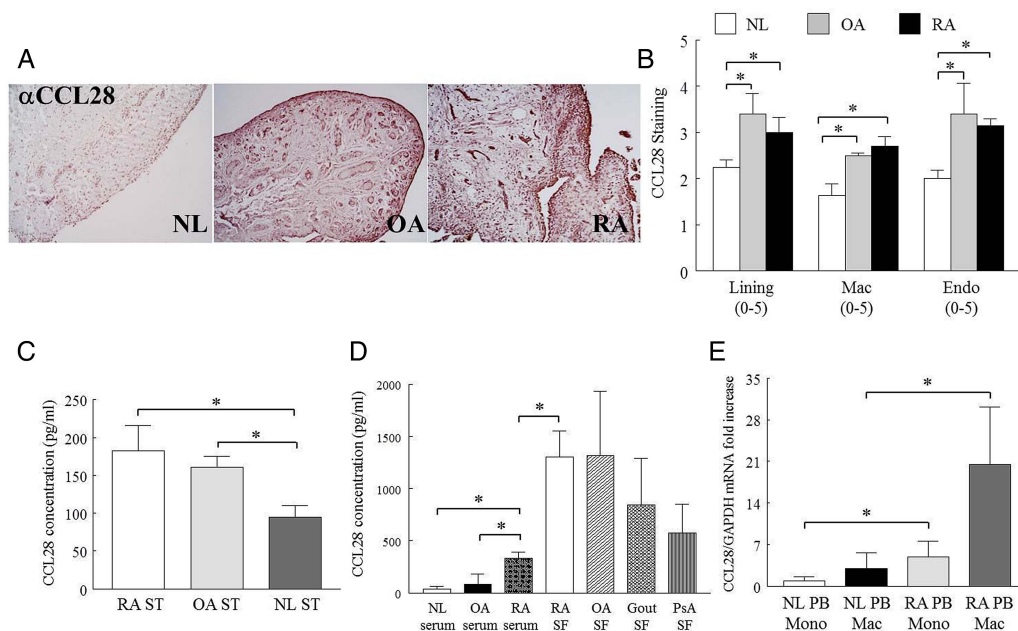
Since both CCL28 and CCR10 are highly coexpressed in RA ST myeloid and endothelial cells, we asked whether their expression levels are regulated in a similar manner. We found that, in RA monocytes and endothelial cells, CCL28 secretion was very responsive to stimulation and was similarly enhanced by toll-like receptor (TLR)4 ligand, TNF- $\alpha$ , IL-1 $\beta$ , IL-17 and IL-6 treatment (figure 3A,C), whereas LPS and TNF- $\alpha$  were the common proinflammatory factors that increased CCR10 protein levels in RA monocytes and endothelial cells (figure 3D,F). Our results further demonstrate that, while stimulation with TLR4 ligand and TNF- $\alpha$  can enhance CCL28 and CCR10 protein levels in RA monocytes and endothelial cells (figure 3A,C,D,F), levels of this ligand and receptor pair are regulated by IL-6 in RA macrophages (figure 3B,E). Consistent with the stimulatory effect of TNF- $\alpha$  on myeloid CCL28 and CCR10 expression, anti-TNF- $\alpha$  therapy shows an insignificant lower trend of CCL28 and CCR10 expression in RA monocytes (see online supplementary figure S2). Taken together, these results suggest that CCL28 and CCR10 protein levels are modulated by an overlapping mechanism of function in RA myeloid and endothelial cells.

### Characterisation of EPCs extracted from NL human blood

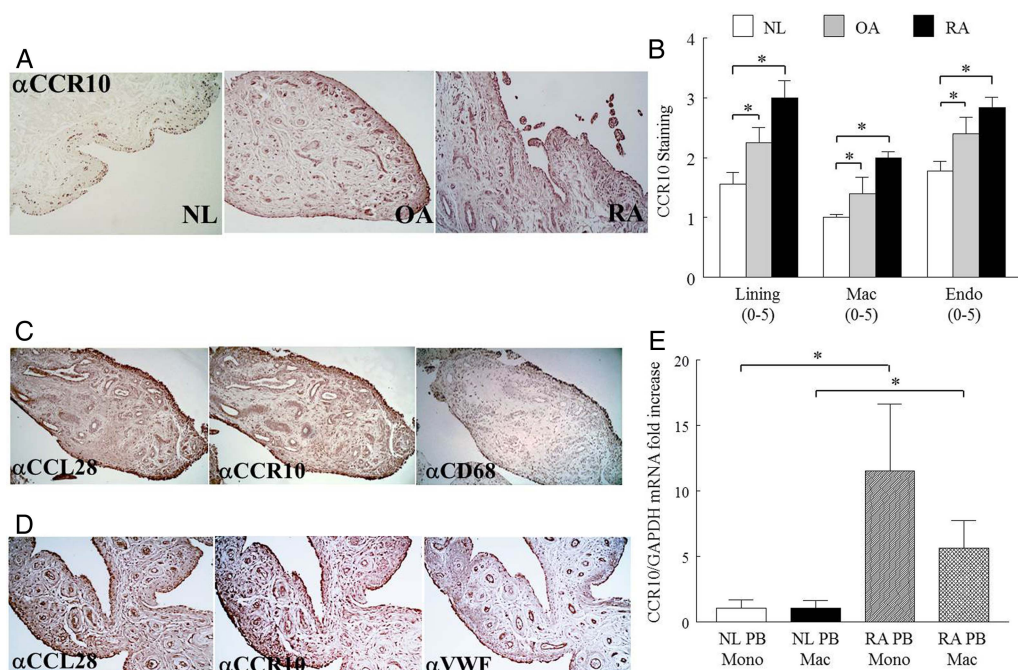
Although RA ST endothelial cells express CCR10, obtaining sufficient quantities of RA endothelial cells from STs is not possible. Furthermore, these cells are not commercially available. Therefore we tested EPCs as possible substitutes for RA endothelial cells. We show that EPCs purified from normal PBMCs were >95% pure based on cells being positive for both VWF and VE-cadherin, while no fluorescence staining was observed in the IgG control group (figure 4G,H). In addition, we document that, similarly to HUVECs, EPCs are capable of forming capillary tubes in response to a proangiogenic stimulus, such as VEGF, and express high levels of CCR10 (figure 4E,F). Hence in this study, human EPCs were used as surrogates for RA endothelial cells.

### Ligation of joint CCL28 to endothelial CCR10 facilitates RA SF-mediated angiogenesis

Since similarly to RA, CCR10 was highly expressed in EPCs and HUVECs (figure 4F), these cells were used as substitutes for RA endothelial cells, and their role was investigated in CCL28-mediated RA angiogenesis. We found that CCL28 strongly attracts endothelial cells starting at a concentration of 0.1 ng/mL, indicating that CCL28 (up to 3300 pg/mL expressed in RA SF) can contribute to endothelial cell migration at a physiologically relevant concentration (figure 4A). We further document that ligation of SF CCL28 to endothelial CCR10 is involved in RA angiogenesis, as neutralisation of CCL28 in RA SF or blockade of CCR10 on HUVECs or EPCs significantly reduces RA SF-driven endothelial chemotaxis or tube formation by 30–40% (figure 4B,C,I). These results suggest that CCL28 at concentrations present in the RA joint can foster endothelial

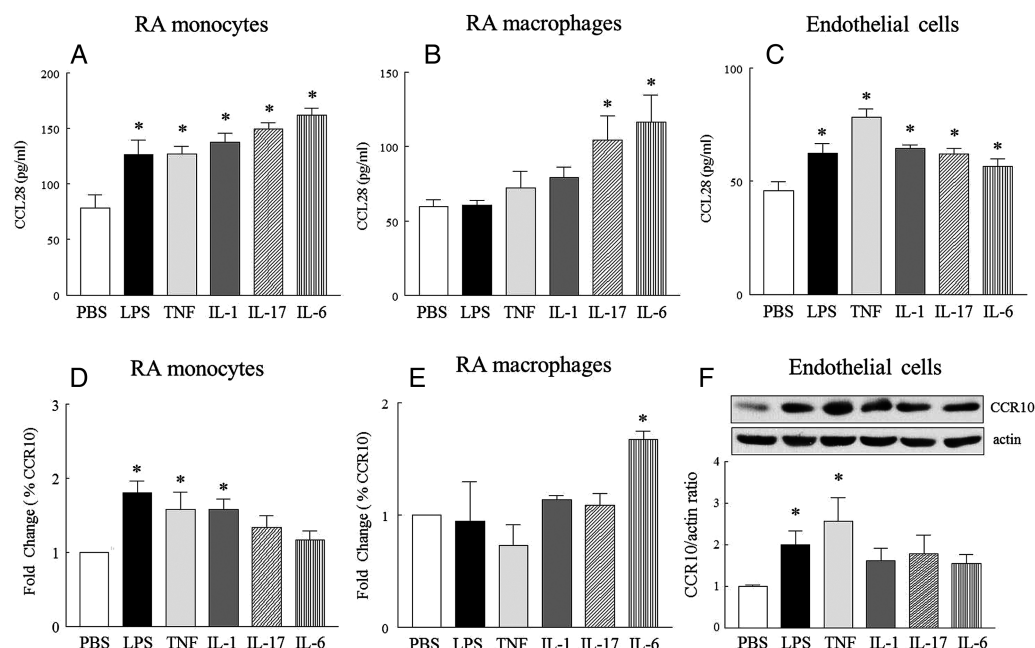


**Figure 1** CCL28 is elevated on rheumatoid arthritis (RA) synovial tissue (ST) myeloid and endothelial cells as well as in RA synovial fluid (SF) and serum. (A) Normal (NL), osteoarthritis (OA) and RA STs were stained with antibody to human CCL28 (1:50) (original magnification  $\times 200$ ). (B) Positive immunostaining was scored on a 0–5 scale in ST lining, sublining macrophages (Mac) and endothelial cells (Endo); staining is shown as mean  $\pm$  SEM (n=10). (C) CCL28 protein levels were quantified in NL, OA and RA STs by ELISA (n=10). (D) Protein concentration of CCL28 was assessed in serum obtained from RA (n=22), OA (n=7) and NL (n=19) and SF from patients with RA (n=22), OA (n=10), gout (n=10) or psoriatic arthritis (PsA) (n=10). (E) Expression levels of CCL28 were quantified in NL and RA monocytes (Mono) and peripheral blood (PB) differentiated macrophages (Mac) by real-time reverse transcriptase-PCR and normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH); the values are shown as fold increase vs NL PB monocytes (n=12–15). Values are mean  $\pm$  SE. \*p<0.05.



**Figure 2** Rheumatoid arthritis (RA) synovial tissue (ST) myeloid and endothelial cells show accentuated CCR10 immunostaining. (A) Normal (NL), osteoarthritis (OA) and RA STs were stained with antibody to human CCR10 (1:50; original magnification  $\times 200$ ; n=10). (B) Positive immunostaining was scored on a 0–5 scale in ST lining, sublining macrophages (Mac) and endothelial cells (Endo); staining is shown as mean  $\pm$  SEM (n=10). Colocalisation of CCL28 and CCR10 on macrophages (CD68+) (C) and endothelial cells (VWF+) (D) was examined when RA serial sections were stained with antibodies to CCL28, CCR10, CD68 and VWF (original magnification  $\times 200$ ). (E) Expression levels of CCR10 were quantified in NL and RA monocytes (Mono) and peripheral blood (PB) differentiated macrophages (Mac) by real-time reverse transcriptase-PCR and normalised to GAPDH; the values are shown as fold increase vs NL PB monocytes and are normalised to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (n=12–15). Values are mean  $\pm$  SE. \*p<0.05.





**Figure 3** Overlapping joint proinflammatory factors can modulate protein levels of CCL28 and CCR10 in rheumatoid arthritis (RA) myeloid and endothelial cells. RA monocytes (A, D) and RA peripheral blood (PB) in vitro differentiated macrophages (B, E) and endothelial cells (C, F) were either untreated or stimulated by 10 ng/mL lipopolysaccharide (LPS), tumour necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$  or IL-6 or 50 ng/mL IL-17 for 24–72 h before determination of protein levels of (A–C) CCL28 by ELISA or (D–F) CCR10 by FACS analysis or Western blotting (n=4–5). (F) In densitometric analysis of CCR10, Western blot bands were normalised to actin using Image J software (n=4). Values are mean $\pm$ SE. \*p<0.05. PBS, phosphate-buffered saline.

infiltration and capillary formation through CCR10 ligation, supporting the importance of the CCL28/CCR10 cascade in RA angiogenesis.

### CCL28-driven angiogenesis is interconnected to ERK signalling

To determine which endothelial signalling pathways are activated by CCL28, phosphorylation of the MAPK and AKT pathways was determined by immunoblot analysis. Our data demonstrate that CCL28 strongly phosphorylates ERK (at 10 and 100 ng/mL), but AKT1, p38 and JNK pathways were not affected by this process (figure 5A, online supplementary figures S3 and S4). To determine which signalling pathway contributes to CCL28-induced endothelial migration and capillary formation, chemical inhibitors at a concentration of 5  $\mu$ M were used, as 10  $\mu$ M was toxic and resulted in cell death. As expected, inhibition of PI3K/AKT, JNK and p38 was ineffective in suppressing CCL28-induced endothelial chemotaxis and tube formation, while inhibition of the ERK cascade markedly reduced both functions by 30–40% (figure 5B–D). These results suggest that joint CCL28 promotes RA angiogenesis through activation of the ERK pathway.

### Activation of the ERK pathway is linked to ligation of CCL28 to endothelial CCR10 and RA angiogenesis

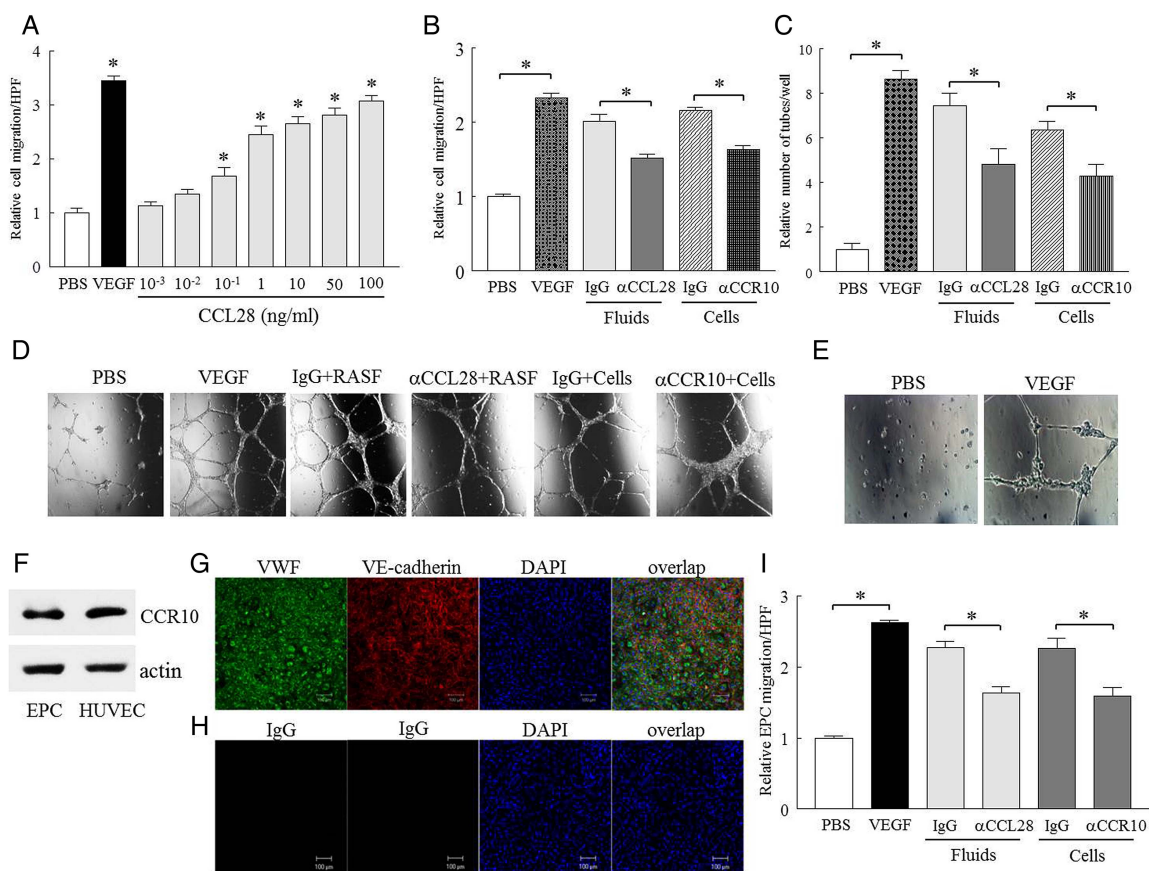
Since phosphorylation of ERK is pivotal for CCL28-induced chemotaxis and tube formation, we next asked whether ligation of CCR10 is interconnected to CCL28 activation of the ERK pathway. Using CCR10-knockdown cells, which had 80% lower CCR10 expression, we found that, while CCL28-driven ERK phosphorylation was abolished compared with control cells, total ERK signalling was unaffected in this process (figure 6A–C). Consistent with this notion, CCR10-knockdown cells showed significantly reduced chemotaxis (40%) and tube formation

(70%) in response to CCL28 compared with the control cells, suggesting that the proangiogenic effect of CCL28 is attributable to CCR10 ligation (figures 6D–F). Collectively, these results suggest that ERK is indispensable for CCR10 ligation of CCL28 and angiogenesis mediated by this factor.

### DISCUSSION

The distribution pattern, regulation and mechanistic function of CCL28 and CCR10 in RA have not been defined. We found that in RA ST, CCL28 and CCR10 are uniquely expressed on myeloid and endothelial cells. Notably, we show that expression levels of this ligand and receptor is similarly modulated by IL-6 in RA macrophages and by TNF- $\alpha$  and TLR4 ligation in endothelial blood vessels and RA monocytes. We reveal that ligation of joint CCL28 to endothelial CCR10 plays an integral role in RA angiogenesis, supporting a novel mechanism of action for the CCL28/CCR10 cascade in RA pathology.

We show for the first time to our knowledge that CCL28 and CCR10 expression is elevated in RA and OA ST compared with NL ST myeloid and endothelial cells. Yet, upregulation of CCL28 has been reported in epithelial cells of inflamed mucosal tissue in the colon, duodenal mucosa and ileum of patients with Crohn's disease and lungs, liver bile ducts and skin of patients with psoriasis and atopic dermatitis.<sup>2 10 27–29</sup> Under normal physiological conditions, CCL28 is predominately produced by epithelial cells present in small and large intestine, reproductive tract, lung, lactating mammary gland and salivary gland as well as by pulmonary dendritic cells.<sup>1 2 4</sup> Moreover, the CCL28 receptor, CCR10, is expressed in T cells, IgA antibody-secreting plasma cells, Langerhans cells and melanocytes.<sup>30 31</sup> Thus the expression profile of CCL28 and CCR10 suggests that they are primarily responsible for regulating immune responses in epithelial-rich tissues.



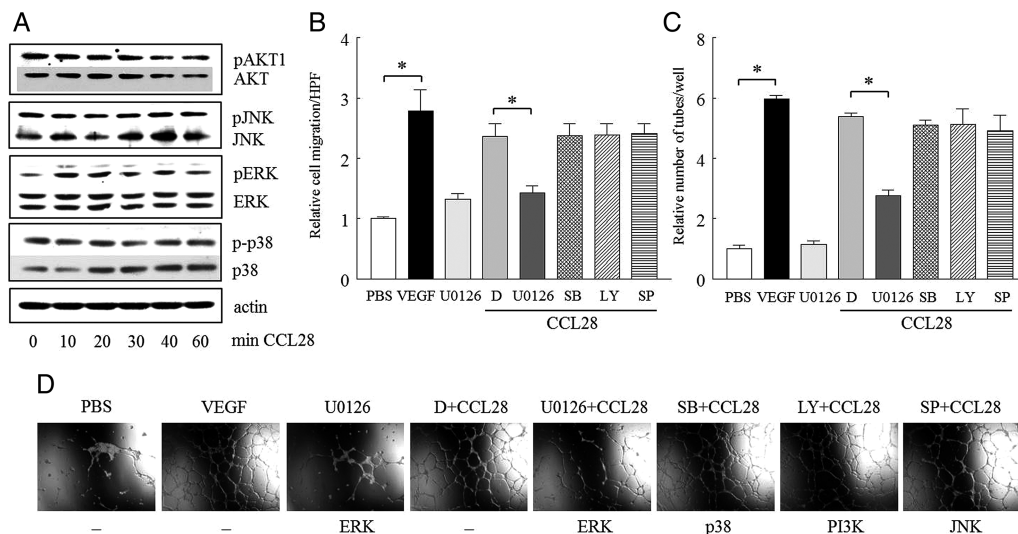
**Figure 4** CCL28 strongly promotes endothelial progenitor cell (EPC) migration and capillary tube formation through CCR10 binding. (A) A dose-response curve of CCL28-induced human umbilical vein endothelial cell (HUVEC) chemotaxis was constructed in a Boyden chemotaxis chamber with varying concentration (0.001–100 ng/mL; n=3). Anti-CCL28 (10 µg/mL) or control IgG was added to rheumatoid arthritis (RA) synovial fluid (SF) (1:20) or HUVECs were incubated with antibodies to CCR10 (10 µg/mL) or isotype control for 1 h before chemotaxis (B) or tube formation (C) in response to RA SFs (n=8). (D) Photomicrographs were taken of representative wells treated with phosphate-buffered saline (PBS), vascular endothelial growth factor (VEGF; 10 ng/mL), IgG or anti-CCL28 (10 µg/mL) plus RA SFs (1:20) and IgG or anti-CCR10 (10 µg/mL) plus HUVECs. (E) The ability of EPCs to form capillary tubes was tested in response to PBS and VEGF (10 ng/mL) (n=3). (F) Expression levels of CCR10 and equal actin loading was assessed in EPCs and HUVECs by Western blot analysis (n=3). (G,H) To validate EPC purity, cells were stained with endothelial markers, von Willebrand factor (VWF; 1:1000), VE-cadherin (1:200) or IgG control, and positive staining was overlapped on 4',6-diamidino-2-phenylindole (DAPI)+ cells (1:1000) (original magnification ×400) (n=3). (I) Anti-CCL28 (10 µg/mL) or control IgG was added to RA SFs (1:20), and EPCs were incubated with antibodies to CCR10 (10 µg/mL) or isotype control for 1 h before chemotaxis (n=3). Endothelial cell chemotaxis or tube formation is shown as a relative value and is normalised to PBS values. Values are mean±SE. \*p<0.05. HPF, high-power field.

Increased levels of CCL28 were detected in ST or SF of arthritic patients (RA, OA, gout and PsA) compared with normal individuals, indicating the importance of the CCL28/CCR10 cascade in inflammation. Although comparable levels of CCL28 were detected in RA and OA ST and SF, CCL28 concentrations were markedly lower in OA than RA sera. Therefore studies were conducted to examine the mechanism by which CCL28 and CCR10 promote pathogenesis in RA myeloid and endothelial cells. Interestingly, we show that differentiated macrophages show a greater trend of CCL28 expression than monocytes, highlighting the significance of CCL28 function in RA joints rather than in circulating blood cells. In contrast, the differentiation process reduces CCR10 expression trend in macrophages. These findings suggest that circulating monocytes may require higher levels of CCR10 to respond to the CCL28 produced by joint cells; however, once these CCR10-positive monocytes reach their destination, the differentiation process results in marginal CCR10 downregulation. In contrast with our observations with CCL28, others have shown that RA monocyte differentiation to macrophages does not alter CXCL16 secretion.<sup>32</sup>

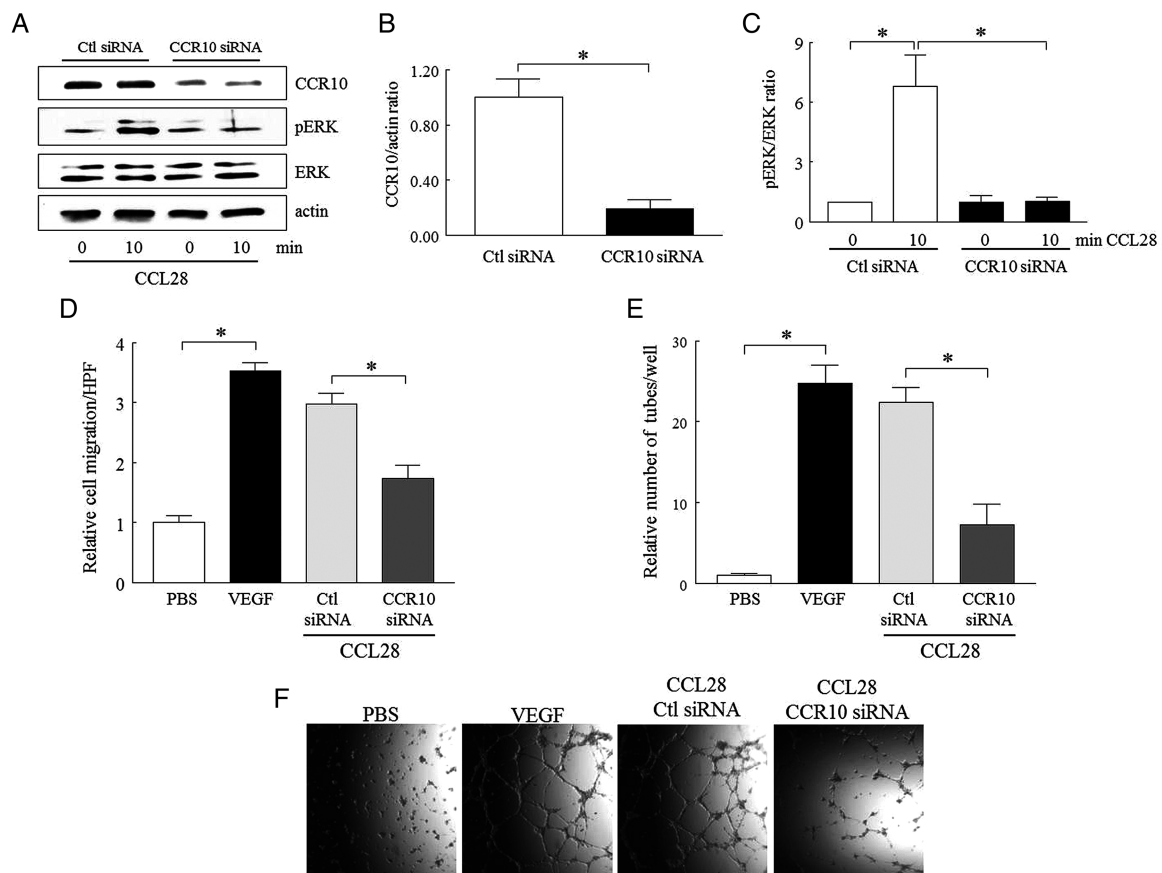
A great number of chemokines (CXCL1, CXCL5, CXCL8, CXCL16 and CCL2, CCL3, CCL5) and chemokine receptors (CXCR1, CXCR2, CXCR4, CXCR5, CCR1, CCR2, CCR5, CCR7, CCR8 and CX3CR1) have been found to be secreted, or are expressed, on RA myeloid cells.<sup>33</sup> Macrophages play a central role in RA pathogenesis, as proinflammatory factors produced by these cells foster monocyte recruitment, neovascularisation and bone destruction, and hence the number of joint myeloid cells correlates closely with inflammation, joint pain and radiological damage.<sup>34 35</sup>

Earlier studies have shown that CCL28 production is modulated by IL-1β and TNF-α in human keratinocytes and by IL-1β, flagellin and/or LPS in the epithelial cells of human colon or bile duct.<sup>3 10 29</sup> Others have shown that IL-17 drives CCL28 production in human airway epithelium.<sup>7</sup> Consistent with these observations, our results show that the same proinflammatory factors, namely LPS, TNF-α, IL-1β, IL-17 and IL-6, provoke CCL28 production in RA monocytes and endothelial cells. However, CCL28 secretion is exclusively modulated by IL-17 and IL-6 in RA macrophages. Like CCL28, expression levels of





**Figure 5** CCL28-driven endothelial migration and tube formation is facilitated by extracellular signal regulated kinase (ERK) activation. (A) Cells were stimulated with CCL28 (100 ng/mL) for 0–60 min and thereafter cell lysates were probed for phospho protein kinase B (AKT1), pJNK (Jun N-terminal kinase), pERK, p-p38 or equal loading controls (n=4–6). Human umbilical vein endothelial cells (HUVECs) were incubated with dimethyl sulfoxide (DMSO) or inhibitors (5  $\mu$ M) to ERK (U0126; with or without CCL28 addition), p38 (SB203580 (SB)), phosphatidylinositol 3-kinase (PI3K) (LY294002 (LY)) or JNK (SP600125 (SP)) for 45 min before endothelial chemotaxis (B) or tube formation (C) in response to CCL28 (50 ng/mL) (n=3). (D) Photomicrographs were taken of representative wells treated with phosphate-buffered saline (PBS), vascular endothelial growth factor (VEGF; 10 ng/mL), U0126, DMSO (D) plus CCL28 (100 ng/mL), U0126 plus CCL28, SB203580 (SB) plus CCL28, LY294002 (LY) plus CCL28 or SP600125 (SP) plus CCL28. HPF, high-power field.



**Figure 6** Angiogenesis fostered through ligation of CCL28 to CCR10 is interconnected to ERK signalling. (A) Human umbilical vein endothelial cells (HUVECs) transfected with CCR10-specific and nonspecific control small interfering (si)RNA (100 nM) were either untreated or treated with CCL28 (100 ng/mL) for 10 min. Thereafter cell lysates were examined for CCR10, pERK (extracellular signal regulated kinase), ERK and actin. Densitometric analysis of the CCR10/actin (B) and pERK/ERK (C) was performed using Image J software (n=3). CCL28 (50 ng/mL)-mediated HUVEC chemotaxis (D) and tube formation (E) was assessed in control and CCR10-knockdown cells (n=3). (F) Photomicrographs were taken of representative wells treated with phosphate-buffered saline (PBS), vascular endothelial growth factor (VEGF; 10 ng/mL), control (Ctl) knockdown cells plus CCL28 (50 ng/mL) and CCR10-knockdown cells plus CCL28. Values are mean $\pm$ SE. \*p<0.05.

CCR10 are influenced by IL-1 $\beta$ , IL-17 and TNF- $\alpha$  stimulation in stromal cells; however, CCR10 is uniquely enhanced by retinoic acids in plasma cells that produce IgA-positive antibody.<sup>36–37</sup> We notably show that TNF- $\alpha$  is the common factor that drives CCL28 and CCR10 expression in blood vessels and RA monocytes, as such RA patients treated with anti-TNF blockers show an insignificant lower trend of CCL28 and CCR10 expression compared with those treated with DMARDs. The fact that CCL28 and CCR10 are modulated by overlapping inflammatory factors in the cell types in which they colocalise makes RA myeloid and endothelial cells very responsive to CCL28 signalling.

The role of CCL28 and CCR10 has been extensively studied in B- and T-cell infiltration, maintenance and immune response.<sup>15</sup> CCL28 plays a highly significant role in homing and preservation of the CCR10+ IgA antibody-secreting plasma cells to mucosal epithelium, since CCR10-deficient mice have an impaired IgA memory response to pathogen reinfection.<sup>9</sup> Further, CCL28 secreted from biliary epithelial cells attracts CCR10+ Tregs, leading to accumulation of Tregs on the mucosal surfaces.<sup>10</sup>

We found that EPCs, similarly to RA joint endothelial cells, express high levels of CCR10 and can form capillaries in response to proangiogenic factors. Corroborating these observations, others have shown that EPCs have a greater ability to migrate into RA ST than NL ST implants, and the number of infiltrated EPCs is markedly elevated in collagen antibody-induced arthritis synovium<sup>38</sup>; therefore EPCs were used as surrogates for RA endothelial cells. Our research team has pioneered the field by demonstrating that the production of CCL28 from joint myeloid and endothelial cells is a strong promoter of angiogenesis in EPCs at a physiologically relevant concentration. We further show that both CCL28 and CCR10 are involved in RA SF-mediated EPC chemotaxis. Moreover, our data document that angiogenesis facilitated through ligation of CCL28 to CCR10 is interconnected to ERK signalling, as CCR10-knockdown cells show dysfunctional CCL28-induced ERK signalling and chemotaxis. To our knowledge, this is the first evidence of a direct role for CCL28 and CCR10 in angiogenesis. A recent study found that, in ovarian tumour cells, hypoxia-triggered CCL28 production promotes homing of CCR10+ Tregs to ovarian tumour.<sup>11</sup> Interestingly, the authors further report that secretion of VEGF from CCR10+ Tregs contributes to ovarian tumour angiogenesis. In this model, Tregs are integral to VEGF production, as anti-CCR10 or anti-CD25 (markers for Tregs) treatment greatly suppresses tumour vascularity and VEGF levels.<sup>11</sup> Although we and others have shown that angiogenesis can be fostered by ignition of the CCL28/CCR10 cascade, there are a number of dissimilarities between these two studies. While we document that CCL28 can directly mediate neovascularisation by attracting CCR10+ endothelial cells, the study by Facciabene and colleagues<sup>11</sup> shows that CCL28 ligation to Tregs is indirectly responsible for tumour angiogenesis.

We have uncovered a novel ligand and receptor pair whose expression has not previously been reported in the cell types identified in this study or in RA. This study unravels the regulation and unique functional mechanism of CCL28/CCR10 in RA, supporting CCL28/CCR10 as a potential therapeutic target in this disease.

**Contributors** Designed the research: ZC, SJK, SS. Performed the research: ZC, SJK, ABE, MVV. Analysed the data: ZC, SJK, MVV, ABE, SS. Provided essential reagents: OMV, WS, SA, SV, NS, LVS. Wrote the paper: all the authors contributed.

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**Competing interests** None.

**Patient consent** Obtained.

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